CREATINE NEUROPROTECTION IN THE RETINA

Paul Ikgan Sia, MBChB

A thesis submitted in total fulfilment of the requirements of the degree of

Master of Philosophy (Ophthalmology)

January 2018

Department of Ophthalmology and Visual Sciences

The University of Adelaide

Declaration

The experiments in this thesis constitute work carried out by me unless otherwise stated. Due acknowledgement has been made in the text to all other material used. The thesis is less than 40,000 words in length, exclusive of tables, figures, bibliography, and complies with the stipulations set out for the degree of Master of Philosophy by the University of Adelaide.

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Paul Ikgan Sia

Adelaide, January 2018

Acknowledgements

This body of work was supported by funds from NHMRC.

Firstly, I would like to acknowledge the contribution of my two supervisors. I am very appreciative to Prof. Robert Casson for providing me this opportunity to undertake Master of Philosophy (Ophthalmology) in the Ophthalmic Research Laboratories of the South Australian Institute of Ophthalmology. Prof. Casson has been a solid constant support as my principal supervisor throughout the past 2 years of my candidature. I also see him as my mentor in this journey of pursuit towards ophthalmology. I would like to thank Dr. John Wood, my co-supervisor, for his excellent supervision in the laboratory. He provided tremendous guidance and help in my experimental designs and analyses of results. He was always there to troubleshoot and solve any problems I encountered during my time in the laboratory. I would also like to thank Dr. Glyn Chidlow for his wisdom and his constructive criticism towards my work.

I thank Teresa Mammone and Mark Daymon for their patience and care in running through with me specific skills that are required in the laboratory. They have also kindly helped out in cutting histological sections and setting up retinal cultures for me when I was heavily short of time. I enjoy very much the times when we solved daily crossword puzzles together and talked about the new Star Wars trilogy. I thank Daniel and Yiani for accompanying me in this journey of ophthalmic research. I cherish very much the time we spent together as peers.

Last, but not least, I thank my parents and brothers for their support and prayers throughout this stressful, but exciting journey.

> "*Unless the LORD builds the house, those who build it labour in vain. Unless the LORD watches over the city, the watchman stays awake in vain*" (Ps 127:1).

Abstract

Studies concerning retinal neurodegeneration have increased remarkably over recent decades as efforts have focused on elucidating mechanisms of damage and ways to halt the degenerative progression. The retina is a complex structure that consists of multiple layers of different types of neurons. The retinal ganglion cell (RGC) layer is particularly susceptible to damage and its degeneration is a feature of conditions such as glaucoma. In addition to well-described mechanisms of retinal neuron injury in glaucoma, such as mitochondrial dysfunction and oxidative stress, energetic failure has also been postulated. In this thesis, emphasis is directed towards investigating the retinal effects of creatine, which has been postulated to act by overcoming tissue bioenergetic failure, in culture and animal models of metabolic dysfunction.

There are two papers to this thesis. The first paper characterizes various markers of RGC in a well-known model of retinal ganglion cell injury – N-methyl-D-aspartate (NMDA)-induced retinal excitotoxicity. As there exists no gold standard marker for RGC for the purposes of neuroprotection studies, we aimed to compare four recent ganglion cell markers by retinal whole-mount immunohistochemistry and observe how they changed over time following NMDA exposure. We found that NMDA-induced RGC injury was maximal within the first 24 hours of exposure and there is good consistency between markers Brn3a, RBPMS and γ-synuclein. Of all markers tested, Brn3a was the most useful marker of RGCs to employ in studies looking at loss and counteraction of loss for these cells. This finding was immediately employed for the in vivo work in the second study.

The second paper addresses the effects of creatine in models of retinal injury both in vitro and in vivo. Firstly, a rat retinal culture model of energetic dysfunction using sodium azide was used. Levels of apoptosis, ATP and reactive oxygen species were also tested. Creatine was found to partially protect cultured

rat retinal neurons from energetic failure, as well as reducing oxidative stress and apoptosis. The study then moved on to in vivo experiments whereby effects of oral creatine supplementation in rat models of NMDA-induced retinal excitotoxicity and retinal ischaemia were examined. RGC reductions were found to be up to 70% but these losses were not significantly reduced by creatine supplementation. When apoptotic levels were assessed, there remained no significant difference between the creatine-fed and control group of rats that underwent NMDA-induced retinal toxicity. Further studies, especially on the mechanisms of creatine neuroprotection, would need to be performed to explain the discrepancy in the ability of this compound to provide neuroprotection in vitro but not in vivo.

Table of Contents

List of Abbreviations

ADP	Adenosine triphosphate
ALS	Amyotrophic lateral sclerosis
ATP	Adenosine triphosphate
CNS	Central nervous system
CK	Creatine kinase
CRT-1	Creatine transporter
DNA	Deoxyribonucleic acid
FBS	Fetal bovine serum
GABA	γ-aminobutyric acid
GCL	Ganglion cell layer
INL	Inner nuclear layer
IOP	Intraocular pressure
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NMDA	N-methyl-D-aspartate
NBF	Neutral-buffered formalin
NTG	Normal tension glaucoma
OCT	Optical coherence tomography
ONL	Outer nuclear layer
PBS	Phosphate buffered saline
POAG	Primary open-angle glaucoma
RBPMS	RNA-binding protein with multiple splicing
RGC	Retinal ganglion cell
SAMe	S-adenosyl-L-methionine
SEM	Standard mean of error
Tdt	Terminal deoxynucleotidyl transferase
TUNEL	TdT-mediated dUTP-biotin nick-end labelling

1 Background and Review of Literature

1.1 Neurodegenerative diseases of the retina

Degeneration of neuronal cells (i.e. neurodegeneration) has been a topic of interest over the recent decades. There is increasing effort in elucidating exact mechanisms of how neuronal cells degenerate in the setting of pathology and how the process may be halted by various agents (i.e. neuroprotection strategies). In the eye, the retina is comprised of multiple types of neurons, namely the retinal ganglion cells (RGC), bipolar cells, amacrine cells, horizontal cells and photoreceptor cells. These neurons are stratified according to location and function, with the photoreceptors taking the role of initiating the process of phototransduction and the retinal ganglion cells being the last cells of the retina to relay electrical signals to the lateral geniculate nuclei and ultimately, the visual cortex.[1-3] Neurodegeneration in the retina can be a result of several conditions, such as glaucoma, diabetic retinopathy, age-related macular degeneration, retinal vascular occlusions or retinitis pigmentosa. The RGCs have been noted to be a main target of degeneration, particularly in conditions such as glaucoma.[4] Therefore, it would be a promising goal to rescue RGCs when managing conditions that lead to retinal degeneration.

1.1.1 RGC structure and susceptibility

The exact cellular constitution and make-up of the retina, especially when it comes to the RGCs, is still being extensively explored. In rodents, up to 32 different functional types of RGCs have been distinguished on the basis of their light responses and basic morphological criteria.[1-3,5] The fact that RGCs have, amongst themselves, different properties is likely due to their upstream connections with bipolar cells that have different properties.[6,7] In primates, RGCs are generally grouped into midget cells (type P; parvocellular), parasol cells (type M; magnocellular) and small bistratified cells. It was observed that the midget cells are responsible for high-acuity vision and they make up approximately half of the total RGC population. The parasol cells, on

the other hand, comprising about 16% of the RGC population, have larger receptive fields, propagate action potentials more quickly in the optic nerve and are more sensitive to low-contrast stimuli. The small bistratified cells have unique roles in encoding blue-yellow colour information. Nonetheless, these major types of RGCs in primate retinae probably make up to roughly three-quarters of the RGC population.[2,3,8-10]

RGCs have long axons that extend through the lamina cribrosa, past the optic chiasm into the optic tract, to the superior colliculus and lateral geniculate nucleus. For this reason, RGCs need high levels of metabolic activity in order to accommodate their extensive energy requirements. Interestingly, compared to large myelinated neurons elsewhere in the body, the axons of RGCs are not myelinated initially, when they are situated within the retina, but only become myelinated once they pass through the lamina cribrosa.[11] Normally, a myelinated neuron would only have lost its myelin sheath only at the very peripheral end of its axon. This leads to an "impedance mismatch" in RGCs – all the more reason for a high metabolic requirement.[11] It can be observed that there are plenty of mitochondria-filled protrusions in the axons of RGCs which are present in order to meet the high metabolic need of these cells. It is also noted that the distribution of mitochondria in the axons of RGCs varies according to location (high numbers prior to passing through the lamina cribrosa), which reflects the differential functional energy requirements of axons and other cellular compartments on the basis of location or region.[12]

These unique structural features of RGCs increase their vulnerability to various potential insults. Despite their high energy requirements, the retinal microvasculature cannot allow a dense coverage due to the need for the inner retinal layer to remain transparent to allow passage of light through to the photoreceptors. Compared to capillaries in other organs, retinal capillaries are very thin and relatively sparse.[13] Along with their long axons, RGCs are therefore vulnerable to receiving metabolic stress (e.g. hypoxia, free radical exposure) along with being susceptible to mechanical compression (e.g. pressure at the lamina cribrosa), and photo-oxidative

damage from light passing through to the photosensitive rods and cones.[14,15] When exposed to this variety of potential stressors, many neuron classes would be unable to remain viable. RGCs, however, are able to compensate by possessing a high antioxidant capacity (endogenous peroxidases). Even despite this being the case, they are still more vulnerable than most cells in their immediate proximity (e.g. Müller and vascular cells).[16]

Functional injury (i.e. in the form of oxidative damage and axonal transport failure) of this very vulnerable population of neurons precedes the onset of structural damage.[17] Therefore, when considering neuroprotective strategies, it would be ideal to intervene at the former stage, prior to the initiation of structural damage. This would more likely allow possible return of function and preservation of the original cellular structure and functioning.[18,19]

1.1.2 Retinal Ganglion Cell Identification

Research into retinal degenerating conditions relies on accurate and reproducible detection of individual cell types. One major challenge, for example, is to reliably distinguish a particular type of neuron from the many different types of cell which may surround it. In the ganglion cell layer, RGCs are situated in amongst displaced amacrine cells, astrocytes, microglia and vascular endothelial cells. Numerous efforts have focused on developing methods to reliably distinguish RGC from the others, especially displaced amacrine cells, which are also neurons.

Traditional histological methods, such as tissue staining with haematoxylin and eosin or Nissl are still used for general tissue assessment, but these techniques largely cannot distinguish different cell types.[20] Since RGCs project to the brain, however, a useful methodology does exist for identification of these cells. Here, a tracer molecule or dye (e.g. Fluorogold)[21] is injected into the brain of a test animal and retrogradely transported back into RGC perikarya and axons in the retina, allowing detection of these cells in tissue whole-mounts. The tracer is administered at main recipient areas for RGCs in the brain (superior colliculi in the case of rodents)

and the retina is examined several days later, allowing time for retrograde transport of the tracer and its accumulation in cell bodies. This method has the benefit of identification of RGC types that project to particular targets in the central nervous system. However, it risks iatrogenic damage to brain and RGC axons, variable uptake of tracer and inadequate labelling of RGCs if the technique is not optimised.[21,22]

Alternatively, RGCs can be identified by immunohistochemistry via detection of proteins or nuclei acids that are specifically expressed by these cells, such as Thy1, NeuN, Islet-1, Brn-3 and Bex1/2.[23-27] The advantage of this method is the ease of the labelling procedure. Furthermore, this method avoids the risk of inadvertent RGC injury prior to immunostaining. Nevertheless, this approach comes with drawbacks. Thy1, a cell surface protein, for instance, changes its expression following retinal damage, thus making it an unsuitable marker for studies on retinal neurodegeneration.[20] There is also a general inadequacy in these markers, compared to Fluorogold retrograde labelling, in detecting the RGC population in its entirety. Despite this, recent studies have provided a number of novel and specific RGC markers which can be labelled on histological sections or whole-mounts. These include transcription factor Brn3a and RNA-binding protein with multiple splicing (RBPMS) with its paralogue, RBPMS2 (*hermes*) and structural protein markers such as γ-synuclein.[20,24-26,28,29]

1.1.3 Glaucoma

Glaucoma refers to a group of ocular conditions united by a clinically characteristic intraocular-pressure associated optic neuropathy with accompanying loss of RGCs.[30] It is estimated that glaucoma will affect more than 80 million people worldwide by 2020 with at least up to 8 million of them becoming bilaterally blind.[31,32] The prevalence of glaucoma correlates highly with age, affecting over 1 in 10 Black people at 80 years of age and 1 in 50 Caucasians between 40 – 60 years of age.[31,33-35] There is no single clinical biomarker to determine the diagnosis of glaucoma, but its presence requires evidence of retinal nerve fibre layer loss (thinning of the retinal ganglion layer), which often is manifest relatively late in the disease

after significant tissue damage has occurred.[36] Indeed, statistically significant visual field abnormalities become noticeable when 25 – 35% of RGC loss has occurred at the corresponding retinal location and the relationship between sensitivity and RGC loss becomes systematic after about 50% of RGCs have died.[35,37] In the context of this condition, it would be ideal therefore if a neuroprotective agent that can deal directly with the pathogenic processes of glaucoma could be administered, either prophylactically or at the time of diagnosis. Here, the clinical features, pathogenic mechanisms and potential treatment of glaucoma, as a condition that classically affects the RGCs, are briefly discussed.

1.1.3.1 Clinical features

Clinically, glaucoma results in optic disc changes where several features can be observed: progressive and asymmetrical cupping of the optic disc, retinal nerve fibre layer loss, optic disc haemorrhage and notching of the neuroretinal rim. In most cases, thinning of the neuroretinal rim of the optic disc is most pronounced in the superior and inferior rim of the optic disc (vertical enlargement of disc cup). This mode of thinning, along with disc pallor, is characteristic of glaucomatous optic neuropathy and is rarely seen in other types of optic neuropathy.[15,35,37-39]

The most common form of glaucoma, primary open-angle glaucoma (POAG) is generally a bilateral disease; however, changes can be asymmetrical. The changes at the optic disc can be translated into characteristic visual field losses, described as nasal step, arcuate scotoma, altitudinal scotoma and generalised depression. In relation to associated high IOP, nasal step and arcuate defects are associated with glaucoma related to elevated IOP, whereas paracentral scotomas are seen more commonly in normal tension glaucoma. Standard automated perimetry and optical coherence tomography of the retinal nerve fibre layers are mainstay investigations used for the diagnosis of glaucoma and monitoring of disease progression. Visual acuity is rarely affected by glaucomatous damage until late in the disease stage.[15,35-37]

1.1.3.2 Mechanisms of retinal ganglion cell injury in glaucoma

The exact pathogenic mechanisms of glaucoma are yet to be elucidated. To date, two major theories predominate: the mechanical and vascular theories.[15,38-43] These theories obviously describe a basis for glaucoma in physical pressure effects and compromised vascular supply, respectively. In reality, pathogenesis is most likely derived from a combination of both of these processes. A critical biophysical component for glaucomatous injury, deriving from both of these theories is the susceptibility of the optic nerve head and/or the retina to IOP-induced forces. The biomechanics of the eye, determined by its anatomy and tissue properties, are affected reciprocally by the IOP, which ultimately influences both blood flow and cellular responses in the retina, especially at the optic nerve head. Insults at the optic nerve head likely initiate RGC degeneration and morphological changes, which translates to the clinical picture of glaucomatous optic neuropathy with visual signs of optic disc excavation and characteristic visual field defects.[44,45] The "type" of insult (or mechanics of the insult) may differ between individuals. Contributions of mechanical and vascular components in each case may vary. Amidst such variation, there may indeed exist pathogenic mechanisms that are yet to be identified.

The mechanical theory proposes that glaucomatous optic neuropathy results directly from pressure-related stresses and strains, independent of the exact level of IOP.[44] We know from practice that raised IOP is sufficient to cause optic neuropathy, as seen in categories of glaucoma that are associated with elevated IOP, such as primary open angle glaucoma, angle-closure glaucoma or secondary glaucoma. It is proposed that the connective tissue that comprises the optic nerve head is highly vulnerable to IOP-related forces, for which damage may occur even at low IOP, provided that IOP-related stresses are sufficient to cause axonal compression at the nerve head. The primary site of axonal damage therefore is thought to be within the lamina cribrosa and the peripapillary sclera. The discontinuity in the scleral shell (at the actual location of the optic nerve head) provides an area where IOP-related stresses are particularly concentrated. Eventually, morphological deformations due

to such stresses in this region cause axonal degeneration and RGC loss (neurodegeneration).[46-48]

At the molecular level, it has been hypothesized that physical 'kinking' of tissue components in the optic nerve head due to elevated IOP causes disturbed axoplasmic flow of, for example, neurotrophic factors and other essential biomolecules. It is also thought that these tissue and cellular defamations ultimately disturb RGCs sufficiently to cause their death via apoptosis.[49] Apoptosis is characterized by deoxyribonucleic acid (DNA) fragmentation, nuclear and chromatin condensation and cell shrinkage – cell clearance in the absence of inflammation. This can lead to activation of glial cells such as astrocytes in the retina which contribute to subsequent tissue remodelling.[50,51] Additionally, metalloproteinases, major matrix-degrading enzymes, are increased in circulating blood of patients with glaucoma. These proteinases may contribute to RGC death and tissue remodelling.[51-53]

The mechanical paradigm for the pathogenesis of glaucoma is unable to fully explain all observed clinical cases, however. Ocular hypertension (elevated IOP at baseline without evidence of optic nerve damage), for example, is more prevalent than actual glaucoma, meaning that not all incidences of elevated pressure convert to the disease.[54,55] In addition, glaucoma can also result from normal IOP (normal tension glaucoma; NTG).[34] This leads to the second hypothesis for the mechanism of glaucomatous tissue injury: the vascular theory. This theory proposes that optic nerve damage from glaucoma is a result of inadequate perfusion, which can be due to elevated IOP or other factors, for example, elevated systemic blood pressure or vasospasm. Whatever causes this vascular insufficiency, the result is a reduction in ocular blood flow and hence, nutrient supply to the tissue.[38,44,56-58]

The blood supply to the optic nerve head can be divided into four zones: superficial nerve fibre layer, prelaminar portion, lamina cribrosa and retrolaminar portion.[59,60] These layers are supplied by small branches of the central retinal artery, branches of the choroidal arterioles, short posterior ciliary arteries and pial vessels.

Blood flow in the retina is characterized by auto-regulated low-flow and subsequent high oxygen extraction. Given that there is no autonomic innervation within the retina, autoregulation occurs through local factors. The choroid, on the other hand, fed by posterior ciliary arteries, is supplied by a non-autoregulated high-flow system with low oxygen extraction. The optic nerve head geographically encompasses blood flow to both the retina and the choroid and the extent of any auto-regulation of blood flow within the area is unclear, although it is thought to be less efficient than that in the retina but better than the choroidal system.[59-62]

In the presence of elevated IOP or reduced ocular blood flow, the ocular perfusion pressure (difference between systemic blood pressure and IOP) declines and supply to the retina is consequently compromised. Accordingly, it has been well observed that patients with diabetes (manifest in eyes as a reduced retinal blood flow secondary to capillary closure or endothelial dysfunction) are found to have a higher prevalence of glaucoma, irrespective of any IOP elevation.[38,56,59,60,62-64]

Reduced retinal blood supply itself theoretically leads to tissue hypoxia and, among other processes, the accumulation of reactive oxygen species. Reactive oxygen species are produced in mitochondria by physiological processes; at low levels these are crucial for cell signalling, however at high levels they lead to cell damage or death via a variety of processes.[53] Oxidative stress, which is defined as the accumulation of reactive oxygen species at pathological levels, has been postulated to play multiple roles in retinal tissue destruction in glaucoma as well as in other neurodegenerative diseases, such as Huntington's disease and Parkinson's disease.[38,40,41,53] Oxidative damage may also affect vascular auto-regulation causing reductions in ocular blood flow and trabecular meshwork damage. Furthermore, it may activate apoptosis via both caspase-dependent and caspase-independent pathways. Oxidative stress also affects retinal glutamate-glutamine cycling by modifying the enzyme glutamine synthetase and causing toxic levels of glutamate to accumulate in the retina; this is termed excitotoxicity and can quickly become retinotoxic. Glutamate, when released into the retinal extracellular space, stimulates

a variety of both ionotropic and metabotropic pre-and post-synaptic receptor. One of the major consequences of this is the stimulation of increases in the concentration of cytosolic sodium ions, and of particular relevance, calcium ions (Ca^{2+}) in neurons. Normally, Ca^{2+} enters intracellular storage sites such as the endoplasmic reticulum or mitochondria. A deficiency in cellular ATP levels as a result of vascular insufficiency means that Ca^{2+} not only rises via uncontrolled glutamate receptor stimulation but via release from intracellular stores. The excessive accumulation of cytosolic Ca^{2+} activates a number of enzymes (e.g. caspases), and signalling pathways, ultimately leading to cell death via apoptosis. Prolonged elevation of cytosolic Ca^{2+} in the absence of adenine nucleotides also results in opening of the mitochondrial permeability pore and this also results in apoptosis. However, it has been shown that cells may remain viable in this state of elevated Ca^{2+} provided that ATP levels remain relatively unchanged.[39-41,43,53,65]

Oxidative stress is also a manifestation of mitochondrial dysfunction. Mitochondria are major generators of reactive oxygen species, with complexes I and III of the oxidative phosphorylation chain being the most active sites of oxygen radical species production. As mitochondria are densely packed in axons around the optic nerve head, simple disruption in either the architecture (compression) or energy metabolism (hypoxia/ischaemia) of the optic nerve head leads to a reduced energy supply for the RGCs. Again, reduced ATP production, or energy failure, is thought to provide a key component in the mechanism of glaucomatous injury.[39,41-43,66]

1.1.3.3 Management of glaucoma

IOP and age are the strongest noted risk factors for the progression of primary open angle glaucoma, with the former remaining the only modifiable risk factor available for treating this disease. Indeed, high myopia, low central corneal thickness and family history of glaucoma are significant but non-modifiable associations with glaucoma. In view of the interest in the vascular theory of glaucomatous injury, risk factors in relation to perfusion abnormalities (cardiovascular disease, systemic hypertension, peripheral vasospasms and migraine headache) have also been

associated with glaucoma, although the evidence for these risk factors being truly involved in the progression of glaucoma is much weaker.[38,56,66]

Clinical management of glaucoma begins with establishment of the extent of glaucomatous optic neuropathy with investigations such as fundus views, visual field testing and optic disc or retinal nerve fibre layer imaging with optical coherence tomography (OCT). IOP can be high or normal in these patients. When diagnosis has been established, IOP reduction is usually performed in a step-wise fashion: starting with IOP-lowering topical eye drops (prostaglandin analogues, beta-blockers, alpha-agonists and carbonic anhydrase inhibitors), and progressing, if necessary to laser therapy (selective laser trabeculoplasty, peripheral iridotomy), filtration surgery (trabeculectomy) and employment of drainage shunts. In a more acute setting, medical systemic therapy may be used (acetazolamide).[67]

There is no clear guideline in deciding the exact target IOP each patient needs following IOP-lowering treatments. In most cases in clinical practice, unfortunately, the decision to lower IOP is only made once visual field changes or retinal damage have been noted. The general consensus, however, for the initial IOP target for a patient starting on treatment is about 1/3 of the patient's initial recorded IOP. Despite this, target IOPs should be individualised on the basis of age, stage of glaucoma and the patient's life expectancy.

Despite the recognition of IOP reduction in glaucoma management, not all optic nerve damage from glaucoma can be avoided (or prevented). This raises the understanding that elevated IOP does not fully account for the pathophysiology of glaucoma and that efforts at establishing viable agents for direct retinal neuroprotection in glaucoma need to be maximised.

1.2 Creatine as a neuroprotective agent

In view of the association between energy failure with increased oxidative stress and subsequent RGC injury, it is worth considering retinal treatment strategies that address the bioenergetics of this tissue. Several bioenergy-based agents have been proposed to date, as potential retinal neuronal protecting treatments. These have included glucose, nicotinamide, coenzyme Q_{10} and creatine.[42] Research from our laboratory has previously demonstrated that experimentally induced hyperglycaemia resulted in significant protection of RGC in models of retinal ischaemia.[68,69] To the best of our knowledge, however, there are no published data directly concerning the effect of creatine in the context of retinal neuroprotection. However, there is evidence that supplementing function of S-adenosyl-L-methionine (SAMe), a precursor molecule in creatine synthesis, has resulted in restoration of photoreceptor function in a rat model of retinal ischaemia, whereby this restoration coincided with increase in creatine transporter (CRT-1) expression following the ischaemia. Expression of CRT-1 is highest in photoreceptor inner segments, photoreceptor perikarya and cone pedicles, as well as retinal ganglion cells. In this study, it was suggested that the recovery of CRT-1 expression, thus presumably its activity, allowed increased creatine synthesis in response to SAMe supplementation and led to a neuroprotective effect in the retina.[70]

Creatine is a guanidine-based compound that is endogenously produced by the liver, kidneys, pancreas and brain, and is taken up into the brain, cardiac and skeletal muscles.[71] There is also evidence that local creatine is preferentially synthesized in the glial cells, e.g., oligodendrocytes, astrocytes, and Müller cells, in the brain and retina.[72] In studies on major neurodegenerative diseases (e.g. Parkinson's and Alzheimer's disease), creatine has been shown to be neuroprotective both in culture and animal studies.[73,74] It has also been shown to be protective in various neuronal injury models, such as NMDA-induced toxicity. It was further proven to provide positive effects to neurons in models of energetic failure resulting from reversible and irreversible inhibitors of succinate-coenzyme Q reductase (also known as

respiratory chain II).[75] In models of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), where the neurotoxin destroys dopaminergic neurons in the substantia nigra to cause symptoms of Parkinson's disease, creatine has shown a dose-dependent neuroprotective effect.[76-78] In transgenic mice models of amyotrophic lateral sclerosis, creatine administration resulted in extension of survival, improved motor performance and reduction in loss of motor neurons.[79,80] Similar results were also shown for transgenic mice models of Huntington's disease, even when administration of creatine was delayed until disease symptoms had been noted.[75,81,82]

Creatine is able to cross the blood-brain barrier into the brain as well as the retina. It exists in the cell as both free creatine and phosphocreatine.[73] An enzymatic process of transferring a phosphate group to creatine to form phosphocreatine is catalysed by creatine kinase (CK), which exists in two isoforms: cytosolic CK and mitochondrial CK (Mi-CK). Cytosolic CK is a heterodimeric protein which consists of two subunits: B (brain) type or M (muscle) type. Three different isoenzymes can therefore result from such combination: CK-MM, CK-BB and CK-MB. There are two tissue-specific Mi-CK isoenzymes, termed sarcomeric Mi-CK (found in striated muscle) and ubiquitous Mi-CK (found in other tissues including neural tissue). Mi-CK, in contrast to the dimeric form of cytosolic CK isoenzymes, forms highly symmetrical, cube-like octameric structures that peripherally bind to lipid membranes and mediate contact site formation between the inner and outer mitochondrial membranes. Cytosolic CK, however, is typically associated with subcellular structures within the cell.[73,83-88]

1.2.1 Creatine Kinase – Phophocreatine system

The 'phosphocreatine circuit' model was proposed to describe how creatine acts as energy buffers in cells with high and fluctuating energy metabolism.[71,89] In general, ATP can be derived from two major synthetic pathways: oxidative phosphorylation and glycolysis. Creatine enters cells via the creatine transporter and while inside the cell it exists either as free creatine or as phosphocreatine. In rats,

creating transporters expressed in retinal capillary endothelial cells are involved in blood-to-retina transport of creatine.[72] A fraction of the cytosolic CK, termed CK-c, acts as a regulatory enzyme to achieve equilibrium between creatine and phosphocreatine and, therefore, between ATP and ADP (Figure 1.1). In this way, creatine acts as a cellular energy buffer. Another fraction of the cytosolic CK, termed CK-g, is coupled to glycolytic enzymes and transfers phosphate groups from ATP produced via glycolysis to creatine, forming phosphocreatine. Mi-CK, on the other hand, interacts with the adenine nucleotide translocator (ANT) of the inner membrane of mitochondria to form a transient dynamic channel at the so-called 'mitochondrial energy transfer contact sites.' ATP generated by oxidative phosphorylation is transported through the ANT in exchange for ADP and is transphosphorylated by Mi-CK to produce phosphocreatine. Phosphocreatine and creatine are transported between the intermembrane space (between inner and outer membranes) of the mitochondria and the cytosolic compartment of the cell via transporter protein porin channels that are situated on the outer membrane. This model of the creatine/phosphocreatine/CK system in essence stresses a functional coupling of ATP production (either via oxidative phosphorylation or glycolysis) with ATP utilization via CK and phosphocreatine, as well as the diffusional capacities of creatine and phosphocreatine. This system is crucial for cells with high energy requirements where creatine and phosphocreatine have taken up roles as energy buffers.[73,83,86]

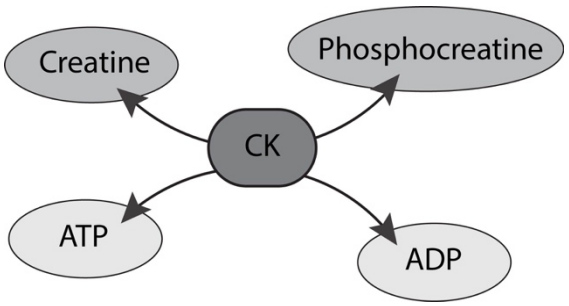

Figure 1.1. Creatine kinase (CK) acts as an energy buffer and provides an equilibrium between creatine and phosphocreatine and, therefore, adenosine triphosphate (ATP)

and adenosine diphosphate (ADP). Diagram shows relationship between creatine/phosphocreatine and ATP/ADP in the 'phosphocreatine circuit.'

1.2.2 Mechanisms of neuroprotection

Creatine may provide neuroprotection to the retina by several mechanisms. In line with the notion that energetic dysfunction plays an important role in the pathophysiology of RGC damage, creatine supplementation may provide protection by increasing cellular levels of phosphocreatine and thus providing greater energy buffering during periods of high metabolic activity as well as for repair processes that occur as a result of injury.[71] Phosphocreatine has a higher diffusion capacity than ATP, which means that transportation of phosphocreatine within the cell serves as a more efficient intracellular energy delivery system. In such situations, the phosphate group attached to phosphocreatine is transferred to ADP for immediate replenishment of ATP. The presence of both cytosolic and mitochondrial CKs further couples areas of energy generation with energy utilization. This CK-phosphocreatine system thus serves as a spatial energy circuit within the cell.[73,83,90-92]

Administration of creatine may also result in a decreased release of glutamate, either through increased glutamate uptake into synaptic vesicles, which is an energy-dependent process, or by an increased conversion of glutamate to glutamine. As a result, glutamate-induced neuronal excitotoxicity (via excessive intracellular Ca^{2+}) may be prevented.[91,93-96]

Creatine supplementation may also prevent apoptosis by inhibiting the opening of mitochondrial permeability pore through the action of Mi-CK.[73,85,91,97] The octameric Mi-CK that is located within the intermembrane space of the mitochondria associates with the mitochondrial permeability pore complex and results in its closure or 'stabilization.' When Mi-CK is converted to its dimeric form, which can happen in the presence of free radicals, its association with the permeability pore is weaker and this leads to opening of the pore. Opening of the mitochondrial permeability pore allows release of apoptosis-stimulating factors and ultimately cell

death. Creatine supplementation prevents octamer-to-dimer conversion by attenuating radical-mediated Mi-CK inactivation.[73,83-85,87]

1.2.3 Animal models of creatine neuroprotection

Creatine was first shown to be strongly protective in a toxic rodent model of Parkinson's disease in 1999.[77] It was shown that intraperitoneal injection of neurotoxin MPTP, which blocks complex I of the mitochondrial respiratory chain, depletes striatal ATP concentrations and causes neuronal damage, but pre-treatment with 1% creatine for 2 weeks almost completely abolished the deleterious effects. Interestingly, this protective effect of creatine was shown to be enhanced in combination with cyclooxygenase-2 inhibitor rofecoxib.[78] In a later study of chronic MPTP intoxication, the combination of creatine and coenzyme Q10 also led to enhanced neuron survival and attenuated tissue damage; this included there being less lipid peroxidation and α-synuclein accumulation within the substantia nigra.[74,75]

In a further study, systemic administration of the respiratory chain complex II inhibitors 3-nitropropionic acid or malonate to rats led to behavioural and neuropathological features resembling human Huntington's disease. When oral creatine was administered before treatment, the treated rats were associated with lower lesion volume and higher brain ATP and phosphocreatine levels.[82] In transgenic mouse models of Huntington's disease, prophylactic creatine administration also provided significant protection by evidence of improvement in motor performance and higher overall survival.[81]

In amyotrophic lateral sclerosis, a standard rodent model uses transgenic mice carrying multiple copies of the human SOD1G93A gene, resulting in early mitochondrial swelling, vacuolization and altered respiratory chain enzyme activities. These changes correspond to similar mitochondrial abnormalities observed in the post-mortem spinal cords of amyotrophic lateral sclerosis patients.[98,99] In these models, two separate studies have reported that 1-2% of creatine, when administered within 1-2 months of birth, resulted in a dose-dependent rise in survival,

reduction of neuronal death, as well as a delay in the manifestation of motor abnormalities, when compared to the placebo.[79,80] The combination of minocycline (tetracycline antibiotic) and COX-2 inhibitors also produced additive neuroprotective effects.[100] Conversely, a separate study, in which common methodological confounders in previously published successful murine SOD-trials were controlled, was only able to report a non-significant survival extension of less than 1% in creatine-treated animals.[101]

1.2.4 Clinical trials of creatine neuroprotection

Given the success in animal models in providing evidence for creatine neuroprotection, several human trials have been performed for major neurodegenerative diseases. A randomized placebo-controlled clinical trial in 2008 found no difference in disease progression in 60 Parkinson's disease patients who were supplemented with creatine over 2 years.[102] In the same year, a futility-monitored, randomized, controlled trial investigating 200 early-onset Parkinson's disease patients concluded that creatine could not be rejected as futile in slowing disease progression and that it therefore showed potential for success in later stage trials.[103] A meta-analysis of two Phase II trials revealed no benefit for creatine on improving motor function in Parkinson's disease patients, but did report that there was perhaps insufficient data to draw any firm conclusions.[104] Interestingly, a separate double-blinded Phase III randomized controlled trial that started in 2007 showed that creatine may even result in more rapid disease progression in Parkinson's disease patients.[105] A recent clinical trial, conversely, investigated the effect of both creatine and coenzyme Q10, in combination, on motor and cognitive functioning and showed that, despite the fact that there was no significant difference in motor symptoms, the cognitive decline in these Parkinson's patients was significantly less pronounced compared to placebo.[106] Regarding the use of creatine for treatment of Huntington's disease, three Phase II randomized controlled trials of premanifest and manifest disease have been published, none of which showed any effect on any clinical outcomes.[107-109] In a further trial of 64 patients with the

premanifest state of Huntington's disease, up to 30g of creatine per day were given to the treatment group. There were no differences in safety and tolerability, although neuroimaging demonstrated a creatine-related slowing of cortical and striatal atrophy at 6 and 18 months.[109]

Taken together, in contrast to animal studies, there has so far been no clear evidence from human trials that creatine can provide significant neuronal protection against any major neurodegenerative disease. It remains unclear why neuroprotection can be observed in animals but not in humans. One proposed reason is the difference in timing of creatine administration between experimental animals and human patients in a clinical setting. In rodent models, neuroprotection has been demonstrated where creatine was administered weeks prior to the initiation of neuronal insult.[75,82] It is obviously unfeasible to take the same prophylactic approach in cases of human neurodegenerative disease, where at the time of symptom onset and diagnosis, the pathological processes have likely been active for a long time. As it is difficult to predict the actual timing of diagnosis, commencement of creatine supplementation in human patients can therefore only be administered when a diagnosis is made. This is true for sporadic neurodegenerative diseases such as Parkinson's disease and amyotrophic lateral sclerosis where creatine administration at the time of diagnosis may well be too late for any neuroprotective effects to surface.[110] In contrast, Rosas and colleagues in 2014 found that Huntington's disease carriers (patients in the premanifest state) receiving creatine had significantly slower rates of cortical thinning compared to placebo-treated individuals, suggesting a potential beneficial effect from creatine on prodromal progression..[109] Although no direct effect of creatine was shown on the actual disease of Huntington's disease itself, it was suggestive that creatine has the potential to slow preclinical progression when administered early. It can also be argued that perhaps the doses of creatine administered in the human trials have not been high enough to show any benefit. Indeed, if creatine was to work in a dose-dependent fashion to protect neuronal tissue, a higher dose of creatine could be provided to patients, especially taking into account the well-described safety profile of this compound. On the other hand, it

may be thought that if creatine is able to provide tissue protection, then some degree of an effect should be observable, for example, a significant slowing in the rate of clinical decline, even when provided after initial diagnosis of the disease, since active pathological mechanisms (e.g. energy failure, oxidative stress and mitochondrial dysfunction) are still in their active state.

Creatine has been associated with increase in nausea and diarrhoea when administered orally, however with no significant clinical or laboratory complications.[109] Safety concerns regarding adverse effects of creatine on renal function, especially if taken at high doses, remain hypothetical. Data from multiple randomized controlled trials for neurodegenerative diseases as well as clinical research fielding other areas of medicine (e.g. myopathies, psychiatric and cardiac disease) have demonstrated that there is a consistently safe profile for the use of externally applied creatine. For example, creatine remains well tolerated even in considerably large doses (up to 30g per day) for months, in elderly participants.[102,108,111]

1.3 Aims and Rationale of Study

Our study was conducted to assess the effects of creatine as a potential protective agent for neurons, including ganglion cells, in experimental situations of retinal cell toxicity. The rationale stemmed from the fact that the RGC degeneration likely occurs via similar mechanisms described for major neurodegenerative diseases elsewhere, in which creatine has shown positive effects. Creatine can be administered orally and has a known good safety profile. It would therefore stand as a promising agent for future retinal disease treatment, if positive effects could be demonstrated in culture or animal studies. In other studies looking at the potential for bioenergetic enhancement as a treatment rationale, researchers have assessed the experimental neuroprotective efficacy of glucose.[42,68,69,112,113] However, although clear demonstration of the protective effects of this compound have been delineated,

issues remain concerning its systemic uptake and whether hyperglycemic microvascular complications may result from its application.

In the first study in this thesis, an immunohistochemical characterisation of the effects to a selection of specific antigenic markers was undertaken in a well-characterized animal model of RGC injury. This study was to provide a greater understanding of the efficacy of specific RGC marker use in a well characterized experimental setting as a prelude to further investigations. As a result of this work, we selected two RGC markers for employment in our subsequent study, in which we investigated the effects of creatine on cell survival in two distinct models of retinal toxicity. This work was supplemented by investigating the protective efficacy of creatine for cells in mixed retinal cultures consisting of dissociated neurons and glia which were subjected to metabolic compromise. Finally, as a result of the findings presented herein, the implications of using creatine for protection of neurons in retinal disease settings in the clinic were discussed.

2 Characterisation of Retinal Ganglion Cell markers in NMDA-induced Retinal Excitotoxicity

2.1 Introduction

The inner rather than the outer retina constitutes the major target of degenerative diseases to retinal neurons other than photoreceptors. In the main, retinal neurons are particularly susceptible to vascular insults, but of these, the retinal ganglion cells (RGCs) are of particular interest in glaucoma. Uniquely, among retinal neurons, RGCs possess long axons which connect with the brain. These projections, however, significantly increase the surface area of RGCs and therefore put them at greater risk of potential mechanical and metabolic insults.[19] In rodents, more than 30 different functional types of RGCs have been distinguished based on their light responses and basic morphological criteria.[1,2] The majority of RGCs share their location in the ganglion cell layer with an approximately numerically equal population of displaced amacrine cells; furthermore, a small proportion have their cell bodies in the inner nuclear layer (known as displaced RGCs).[1,2] Simple identification of cell types according to their position within different retinal strata, thus, is not sufficient. The challenge, therefore, in this area of research is to be able to specifically identify individual cell types, i.e. RGCs and to distinguish them from all other local cells.

A well-established method of RGC identification is via retrograde tracer labelling. Here, a suitable cell label is applied to the main recipient areas for RGCs in the brain, e.g. the superior colliculi, in rodents, and as this is retrogradely transported back into the retina, it will appear in RGC bodies, thus allowing their identification.[114] Fluorogold has been used as a tracer of choice in visual research as it can actively be transported back from axons to RGC cell bodies and accumulates without leaking.[21,22] In studies where application of fluorogold was applied to the optic nerve or tract, all RGCs were labelled after 3-7 days.[21,22,114] When applied to both superior colliculi, up to 98% of RGCs are labelled in rodents. However, in order to gain access to the superior colliculus, complex surgery and fine tissue manipulation are necessary.

These could either risk damaging the brain, leading to significant cerebral inflammation, or insufficiently label RGCs if techniques were not optimal.[22,114]

An alternate approach to RGC identification is via immunohistochemical detection of proteins or nucleic acids specific to these cells. For instance, in the context of the physiological retina, Thy1 has been described as an RGC-specific antigen.[115,116] This is of use for detection of RGCs in control tissues, however the pattern of Thy1 expression changes after retinal damage, with this antigen being subsequently detected in other local cells to a small degree, such as Müller cells, amacrine cells or bipolar cells.[117] Labelling of Bex1/2 provides visualisation of both RGC bodies and their axons, allowing good morphological use, but would not be suitable for quantification purposes.[118,119]

For quantification of RGC loss or damage in diseased retinas and relevant model systems, there is, therefore, a great need for identification of a suitable, specific marker of these cells. Immunohistochemical labelling of RGCs in retinal whole-mounts rather than sections is preferable as this method covers the distribution of these cells throughout the retina, rather than in just a single dimension. Quantification of RGCs on whole-mount preparations therefore provides higher reliability and accuracy in estimating true numbers of these cells. Several RGC markers have been proposed as reliable RGC markers on whole-mounts, including Brn3a, γ-synuclein and RNA binding protein with multiple splicing (RBPMS).[23,25,28,29,118,120] Calretinin also labels cells in the retinal ganglion layer, although it also labels positive for cells in the nuclear layer.[121] Despite the availability of all of these potential RGC marker antigens, there is a paucity of data available which compares their labelling efficiency and their use in quantifying cell loss, especially in rodent models. Our study aimed, therefore, to employ a spatio-temporally well-characterized model of RGC loss, namely N-methyl-D-aspartate (NMDA)-induced retinal excitotoxicity, and compare the use of different RGC markers as a means of quantifying the loss of these cells.

2.2 Methods

Animals

Adult Sprague-Dawley rats were obtained from the University of Adelaide, Adelaide, South Australia (SA). The use of animals was approved by the Animal Ethics Committees of SA Pathology/Central Adelaide Local Health Network (CALHN) and the University of Adelaide. Procedures were performed in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 2004, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were housed with standard food and water provided ad libitum. The light was turned on at 7 AM and turned off at 7 PM. Animals were divided into groups randomly and the eyes were also randomized for procedures. Topical ophthalmic ointment (chloramphenicol) was applied immediately following as well as 12 hours after procedures whenever able.

Retinal Excitotoxicity Model

Intravitreal injection of NMDA was used to induce RGC loss in rat retinas according to previously described method.[122-124] Briefly, 9- to 10-week-old Sprague-Dawley rats were anesthetized by inhalation of isoflurane (4.0 – 5.0%) in oxygen. After application of topical tetracaine (0.5%), an intravitreal injection of 5 µL of 4 mM (corresponding to 20 nmol) NMDA (Sigma-Aldrich Corp) in 0.9% sterile saline solution was administered to each eye. The animals were euthanized at 8 hours, 24 hours, 3 days, and 7 days after injection via transcardial perfusion with 0.9% saline solution. Some animals were randomized to a control group where NMDA was not administered. Their eyes were enucleated for subsequent analyses (n = 6-7 per group).

Whole-Mount Immunohistochemistry of Brn3a, γ-synuclein, calretinin, and RBPMS

The enucleated globes were immersed in 10% buffered formalin for 24 hours before being dissected and flat-mounted on cellulose nitrate filters (pore size 60 µm).

Retinal whole-mounts were washed with PBS before being permeabilised with PBS containing 3% Triton-X, and subsequently blocked in PBS containing 1% Triton-X and 3% normal horse serum. Standard immunohistochemical procedures were performed with primary goat anti-Brn3a (1:600), mouse anti-γ-synuclein (1:100), rabbit anti-calretinin (1:300), and rabbit anti-RBPMS (1:300) antibodies. Alexa Fluor 594 and 488 anti-goat, anti-mouse, and anti-rabbit secondary IgG antibodies (1:250, Molecular Probes) were used for double fluorescence labelling. Briefly, the whole-mounts were incubated with primary antibody in PBS containing 1% Triton-X and 3% normal horse serum overnight at 4˚C and then secondary antibody for 3 hours at room temperature. Tissue preparations were then washed in PBS before being mounted using anti-fade mounting medium (DAKO, Botany, New South Wales, Australia) and finally, sections were examined under a confocal microscope (Olympus) with fluorescence optics (Zeiss Australia, Sydney, Australia).

Cross-sectional Immunohistochemical Analysis

Enucleated globes were immersed in 10% buffered formalin for 24 hours and processed for routine paraffin-embedded sections. Globes were embedded sagittally and 5-µm serial sections were cut. Standard immunohistochemistry was performed as previously described. In brief, tissue sections were deparaffinised and treated with 0.5% hydrogen peroxide to block endogenous peroxidase activity. Antigen retrieval was achieved by microwaving the sections in 10 mM citrate buffer (pH 6.0) for 10 minutes at 95˚C to 100˚C. Tissue sections were then blocked in PBS containing 3% normal horse serum, incubated overnight at room temperature in primary antibody and subsequently exposed to consecutive incubations with biotinylated secondary antibody and streptavidin-peroxidase conjugate. Colour development was achieved with 3’-,3’-diaminobenzidine. Sections were typically counterstained with haematoxylin, then dehydrated and mounted.

Quantification of RGCs in Retinal Whole-Mounts

For Brn3a and RBPMS counts, eight rectangular areas (2 central, 3 middle, and 3 peripheral) at 1 mm, 2 mm, and 3 mm from the optic disc were analysed for each retinal quadrant yielding 8 x 4 separate retinal areas for RGC quantification (Figure 2.1). For calretinin and γ-synuclein counts, the central areas were omitted as the presence of highly apparent nerve fibres prevented adequate RGC quantification. Each rectangular area measured 0.722 mm x 0.407 mm. Quantification was obtained by a combination of automated and manual counting with ImageJ software by an observer who was masked to the group allocation. Figure 2.2 shows representative images of each RGC label used and the size and morphology of cells included for quantification. To exclude the possibility that antibody labelling was detecting displaced amacrine cells which constitute roughly 50% of the ganglion cell layer, cells with a diameter of less than 10µm, i.e. unlikely to be ganglion cells, were excluded, particularly in the case of calretinin and γ-synuclein. This is because amacrine cells are less likely to be directly affected by NMDA and therefore their presence would introduce bias to the data, depending on the extent of labelling of these cells by each antibody.[125-128]

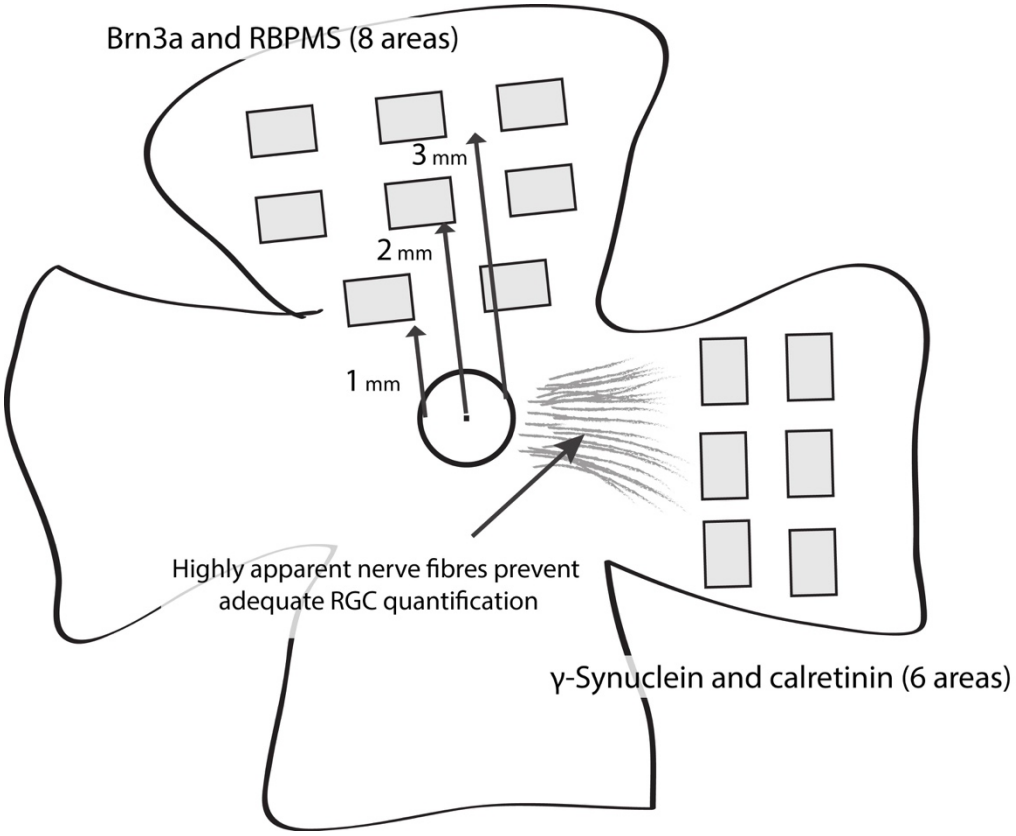

Figure 2.1. Schematic diagram showing area selection for RGC quantification. Eight rectangular areas (0.722 mm x 0.407 mm each rectangle) for each retinal quadrant were sampled for Brn3a and RBPMS quantification. Six areas were selected (middle and periphery) for γ-synuclein and calretinin due to the high density of nerve fibres in the areas adjacent to optic nerve that prevents adequate retinal ganglion cell quantification. *RBPMS*, RNA binding protein with multiple splicing; *RGC*, retinal ganglion cell.

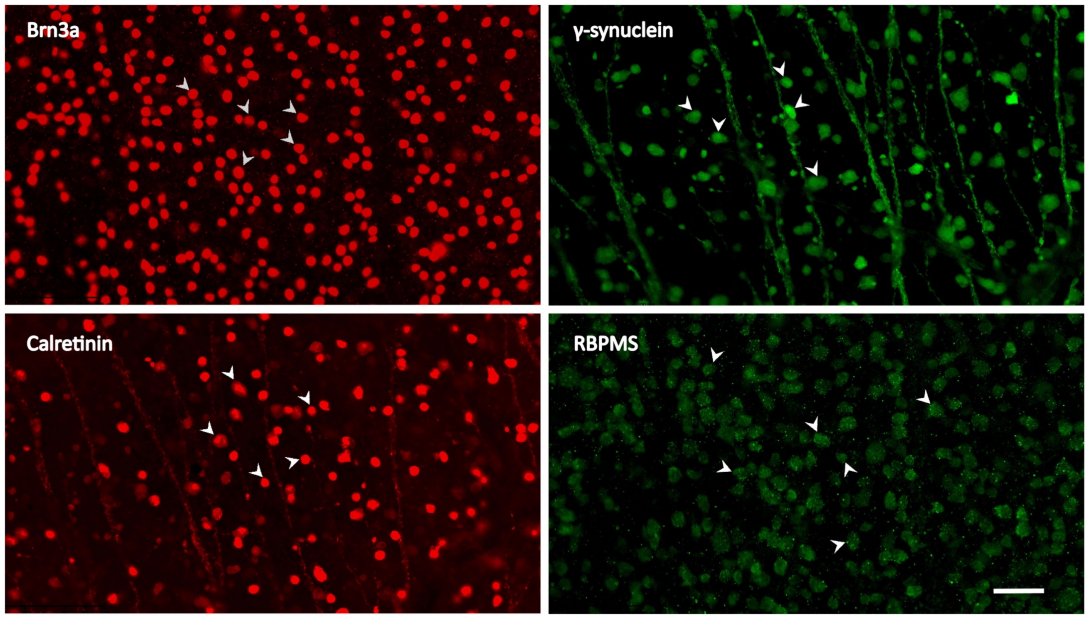

Figure 2.2. Immunofluorescence photomicrographs showing RGCs in whole-mounted retinas stained by four different markers: Brn3a, γ-synuclein, calretinin and RBPMS. Arrowheads refer to examples of cells that were assessed for quantification purposes (For γ-synuclein and calretinin, cells/labels smaller than 10μM were not included for quantification). Scale bar = 50 μm.

Statistical Analysis

Data were presented as the mean percentage of control ± standard mean of error (SEM). Differences among groups were analysed by one-way ANOVA, followed by the Tukey multiple-comparison test. *P* less than 0.05 was considered statistically significant.

2.3 Results

Application of NMDA caused a significant reduction in numbers of RGCs as observed by quantification of all markers of interest (Brn3a, γ-synuclein, calretinin and RBPMS). Cell loss was consistent with previously published data.[124,128,129] Figure 2.3 and Table 1 show RGC loss as assessed by the 4 RGC markers across 5 data points (untreated control and 8-hour, 24-hour, 3-day and 7-day post-NMDA injection), expressed as percentage of remaining immunoreactivity with regards to the untreated control. Data for all other the figures are instead expressed in raw counts with statistical analyses performed (ANOVA with post-hoc Tukey multiple-comparison test).

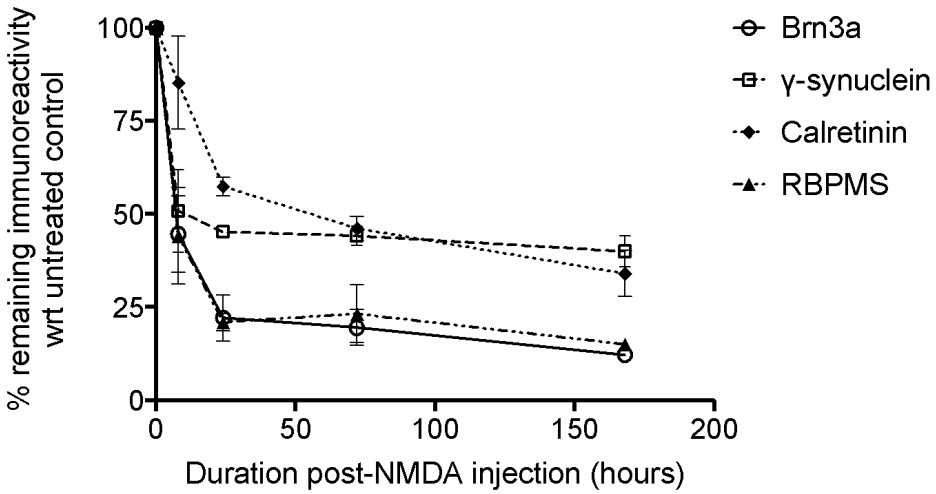

Figure 2.3. Percentage remaining immunoreactivity for Brn3a, γ-synuclein, calretinin and RBPMS across different data points (Control – 0 hour, 8-hour, 24-hour, 3-day and 7-day post-NMDA injection).

Table 1. Percentage remaining immunoreactivity for Brn3a-, γ-synuclein-, calretinin- and RBPMS-positive RGCs over time post-NMDA treatment

RGC label	**Remaining immunoreactivity in % wrt Controls**				
	CONTROL	8-hour	24-hour	3-day	7-day
Brn3a	100 (n = 5)	44.6 ± 10.3 (n = 5)	22.1 ± 6.2 (n = 5)	19.5 ± 4.9 (n = 5)	12.2 ± 1.6 (n = 7)
γ-synuclein	100 (n = 5)	50.8 ± 11.1 (n = 5)	45.3 ± 0.8 (n = 7)	44.2 ± 2.6 (n = 7)	40.0 ± 4.2 (n = 6)
Calretinin	100 (n = 5)	85.2 ± 12.5 (n = 5)	57.4 ± 2.5 (n = 5)	46.2 ± 3.3 (n = 6)	34.0 ± 6.2 (n = 5)
RBPMS	100 (n = 5)	44.2 ± 13.0 (n = 5)	21.0 ± 2.3 (n = 5)	23.2 ± 7.9 (n = 5)	15.1 ± 1.0 (n = 5)

Brn3a

Figure 2.4 shows photographic representations of Brn3a immunolabelling of retinal wholmounts from all time points (Fig. 2.4a – e) along with a graphical summary of averaged raw counts (Fig. 2.4f). At 8 hours and 24 hours following NMDA injection, multiple 'shrunken' (pyknotic) Brn3a-positive cells were noted beside healthy Brn3a-positive cells (mainly Fig. 2.4c, 24-hour time-point), likely denoting peak timing for NMDA-induced apoptosis. There is also an observed 'time-dependent' reduction in Brn3a-positive cells. This is also evident when quantification is stratified on the basis of location (Fig. 2.5a; central, middle, peripheral).

Quantification revealed that Brn3a-positive cells at all analysed times after NMDA treatment were significantly reduced when compared with control retinas (Fig. 2.4f; P = 0.001). Similarly, Brn3a counts at the 8-hour time-point were significantly higher than both the 3-day and 7-day time-points (Fig. 2.4f; P = 0.03 and 0.002, respectively), when taking into account all retinal regions at once. When separating data into separate retinal regions, the cell loss profiles were similar across all three regions (central, middle and peripheral).

Figure 2.6 shows immunohistochemical cross-section staining with Brn3a of retina from control eyes and eyes at 8-hour, 24-hour, 3-day and 7-day post-NMDA treatment.

RBPMS

Comparing quantification of RBPMS-positive cell counts at all data points, there was a similar trend to Brn3a, with statistical difference found when comparing counts from each NMDA time point and the untreated controls (Fig. 2.7; P = 0.001). This was also the case when the central, middle and peripheral parts of the retina were examined separately and individually compared between different treatment times and controls (Fig. 2.5; P = 0.001). In the photomicrographs of RBPMS at 8 hours and 24 hours post-NMDA, multiple 'shrunken' RBPMS-positive cells could also be noted, as was the case with Brn3a. Although the raw counts differed from counts for Brn3a positive cells, numbers of RBPMS-labelled cells exhibited a very similar downward trend when data was expressed as percentage of control counts (Fig. 2.3).

Figure 2.8 shows immunohistochemical cross-section staining with RBPMS of retina from control eyes and eyes at 8-hour, 24-hour, 3-day and 7-day post-NMDA treatment.

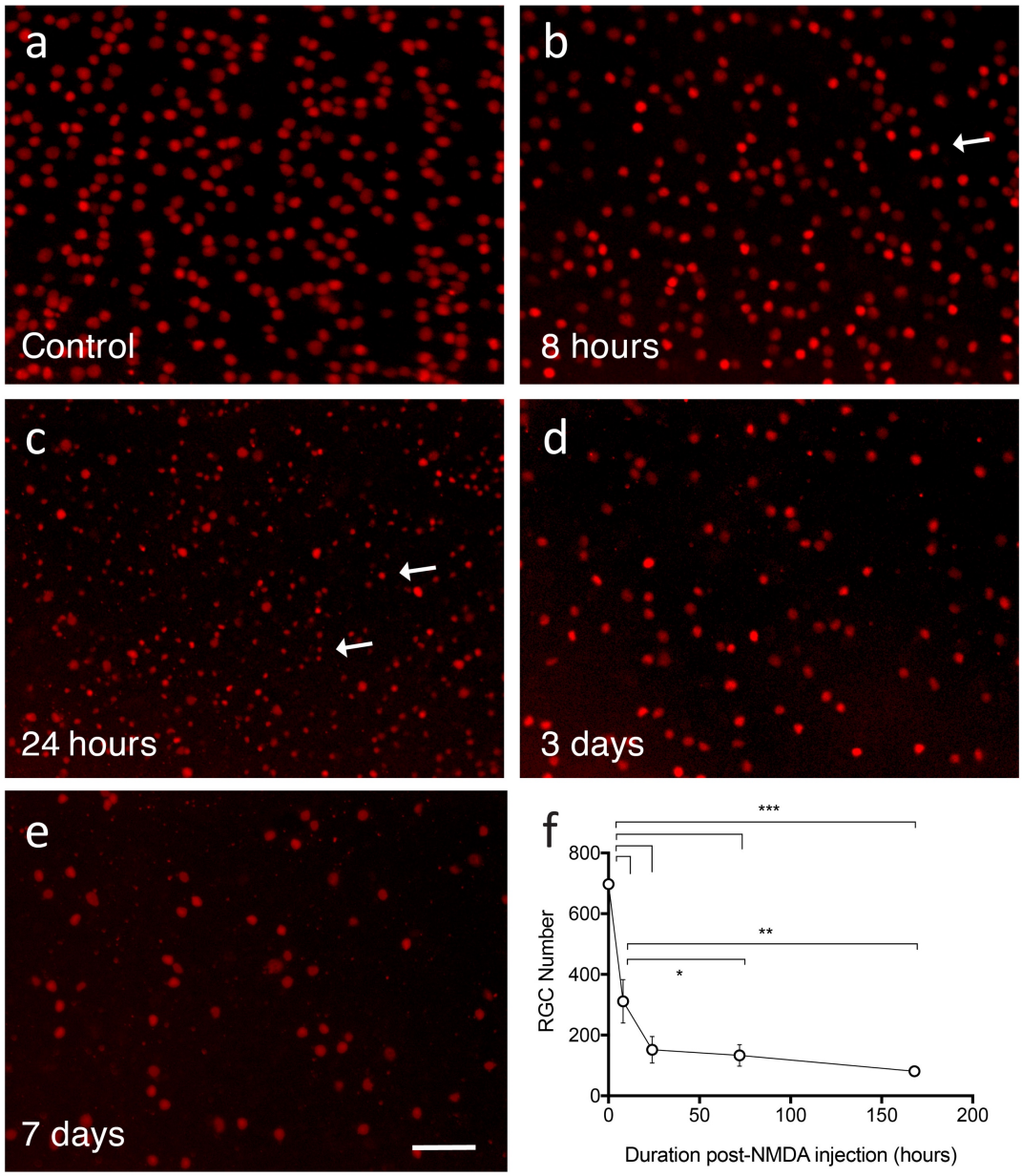

Figure 2.4. (a – e) Photographic representations of Brn3a-positive cells from all time points (Control, 8 hours, 24 hours, 3 days and 7 days) and (f) graphical summary of averaged raw counts of Brn3a-positive cells over time after NMDA exposure (n = 5-7). Arrows point to pyknotic apoptotic nuclei. *P < 0.05, **P < 0.01, ***P < 0.001 by one-way ANOVA test followed by a Tukey multiple-comparison test. Scale bar = 50 µm. *RGC*, retinal ganglion cell.

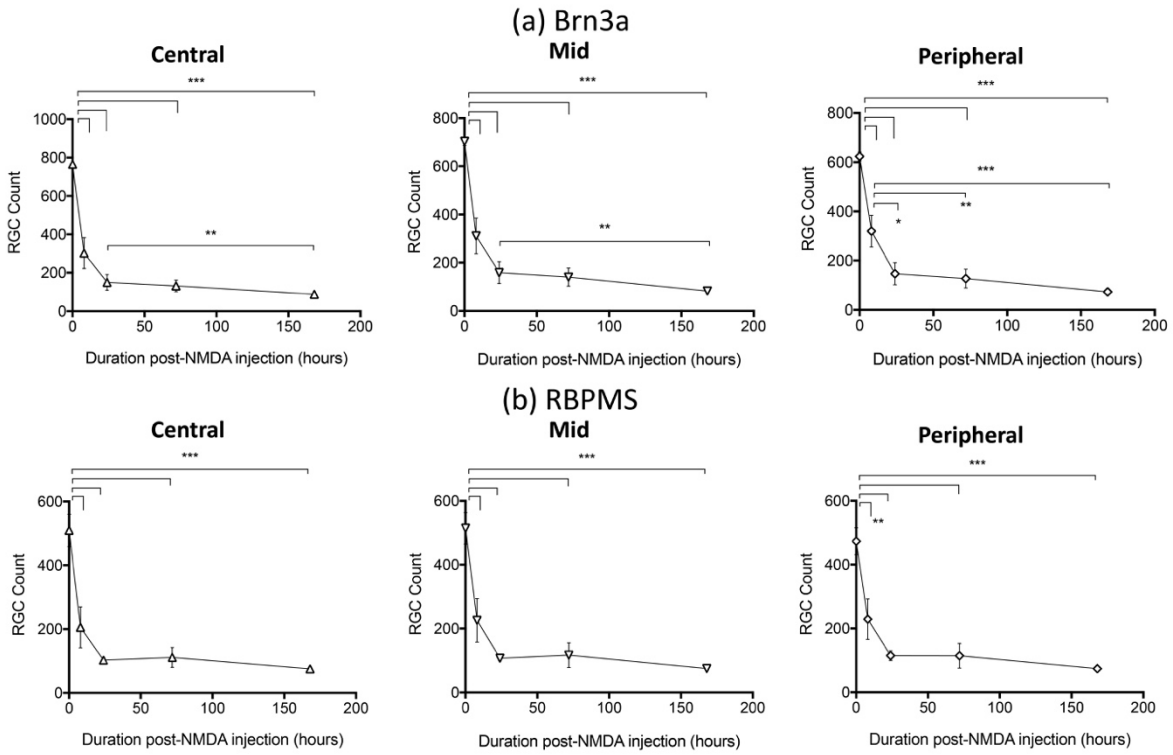

Figure 2.5. Averaged raw counts for central, mid and peripheral areas of the retina in (a) Brn3a-positive and (b) RBPMS-positive cells plotted versus time after NMDA exposure (n = 5-7). *P < 0.05, **P < 0.01, ***P < 0.001 by one-way ANOVA test followed by a Tukey multiple-comparison test. *RGC*, retinal ganglion cell.

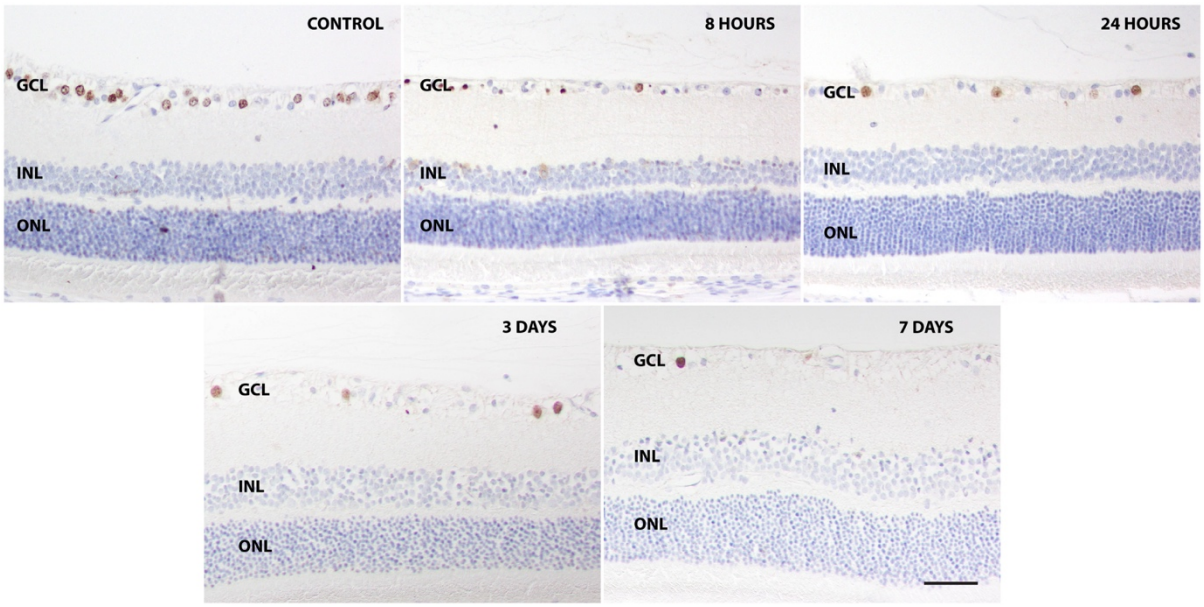

Figure 2.6. Histological cross-section of central retina in eyes treated with NMDA injection. Retinal staining with anti-Brn3a showing progressive loss of retinal ganglion cells for control eyes and eyes harvested at 8 hours, 24 hours, 3 days and 7 days after NMDA injection. Scale bar = 100 µm. *GCL*, ganglion cell layer; *INL*, inner nuclear layer; *ONL*, outer nuclear layer.

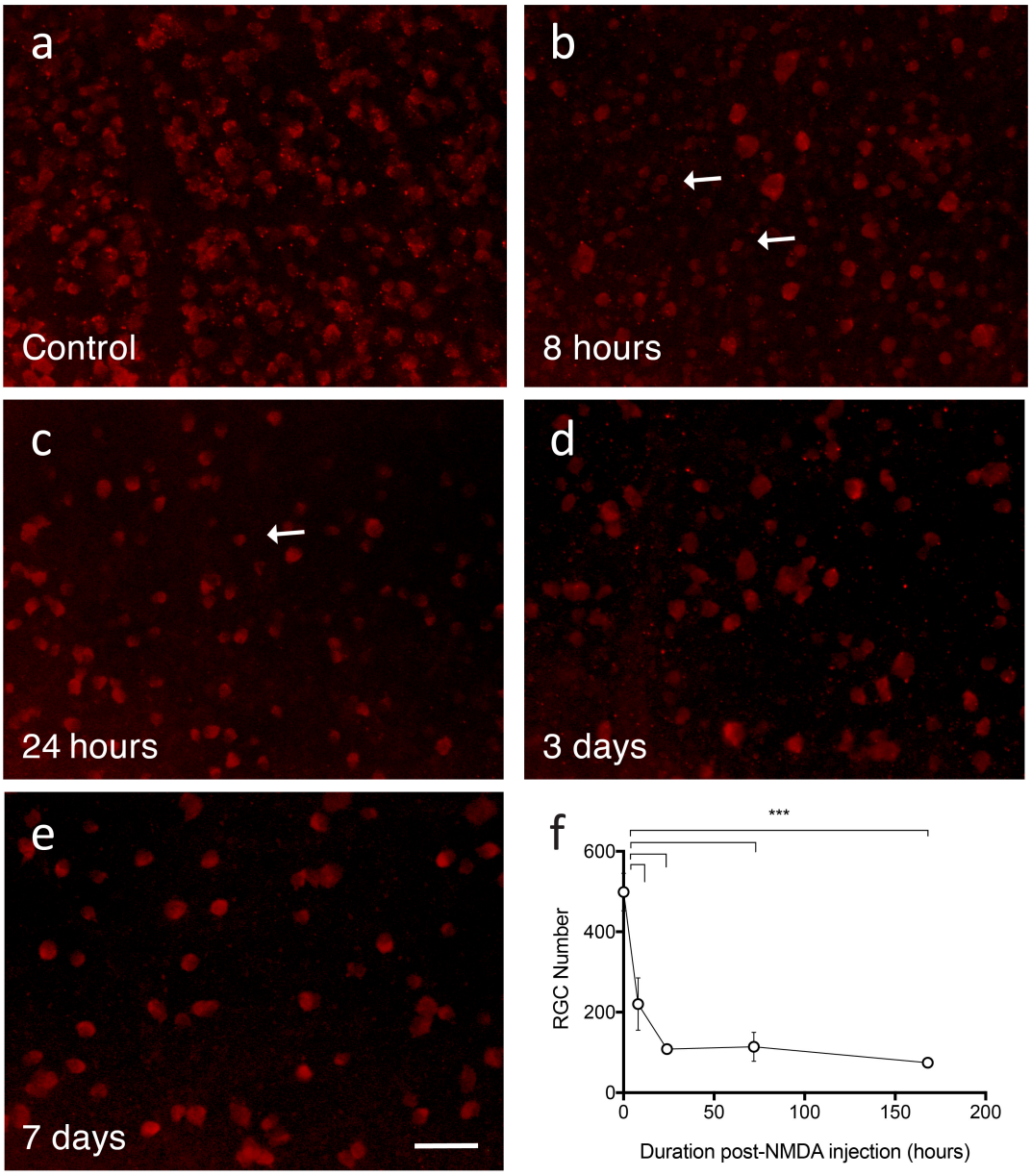

Figure 2.7. (a – e) RBPMS-positive cells at all time points (control, 8 hours, 24 hours, 3 days and 7 days) and (f) graphical summary of averaged raw counts for RBPMS-positive cells over time after NMDA exposure (n = 5). Arrows point to pyknotic apoptotic nuclei. *P < 0.05, **P < 0.01, ***P < 0.001 by one-way ANOVA test followed by a Tukey multiple-comparison test. Scale bar = 50 µm. *RGC*, retinal ganglion cell.

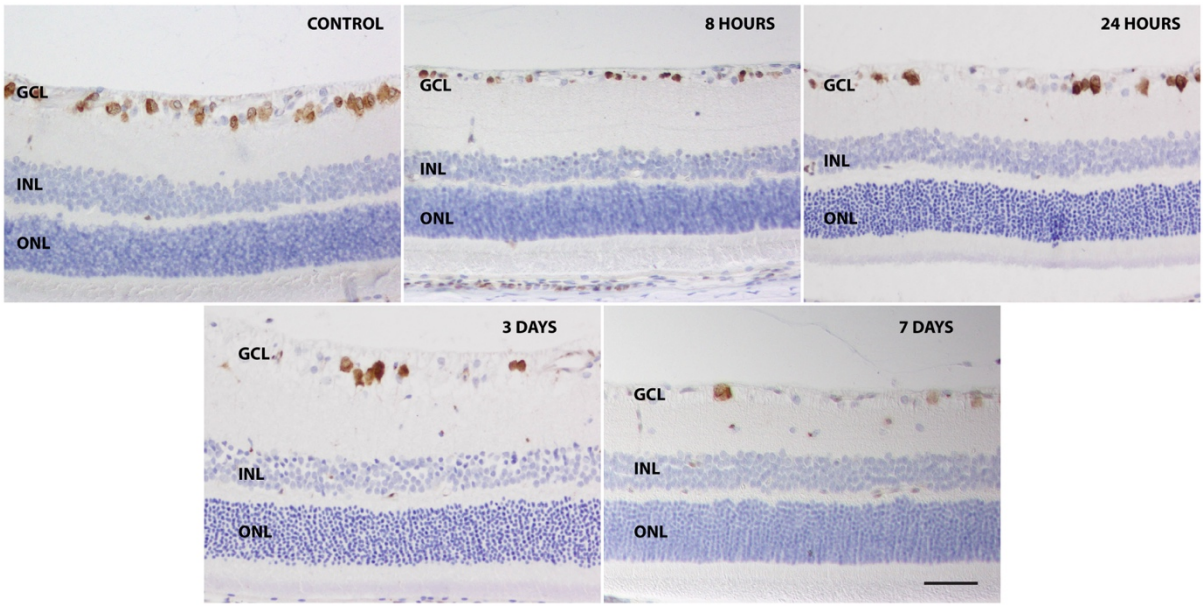

Figure 2.8. Histological cross-section of central retina in eyes treated with NMDA injection. Retinal staining with anti-RBPMS showing progressive loss of retinal ganglion cells for control eyes and eyes harvested at 8 hours, 24 hours, 3 days and 7 days after NMDA injection. Scale bar = 100 µm. *GCL*, ganglion cell layer; *INL*, inner nuclear layer; *ONL*, outer nuclear layer.

γ-synuclein

When assessing γ-synuclein-positive cells, it was obvious that there was a similar decline in numbers as seen with RBPMS, up to 8 hours after NMDA injection; thereafter the rate of cell loss was reduced and the number of labelling cells was approximately static for the remainder of the experiment (Fig. 2.3; Fig. 2.9). There was a significant difference between cell numbers in the untreated retina compared with each of the NMDA-treated time points (Fig. 2.9; Fig. 2.10; P = 0.001). This was the case for total cell numbers from across the whole retina and when comparing data for the middle and peripheral regions (Fig. 2.9; Fig. 2.10; P = 0.001).

Figure 2.11 shows immunohistochemical labelling of retinal sections for γ-synuclein in control, untreated and in NMDA-treated eyes at 8-hour, 24-hour, 3-day

and 7-day post treatment. Labelling of γ-synuclein is present in cells in the ganglion cell layer as well as in some cells in the outer part of the inner nuclear layer which resembled horizontal cells.

Calretinin

Positive labelling for calretinin demarcated cells in 2 distinct retinal strata – the RGC layer and the inner nuclear layer. Figure 2.12 shows calretinin-positive cells with associated nerve fibers in the retinal ganglion layer (Fig. 2.12a-e). Compared to control, reduction in calretinin-positive cells due to NMDA treatment only started to be statistically significant from 24 hours (Fig. 2.12f; $P = 0.02$ for counts between control and 24-hour NMDA, $P = 0.001$ for counts between control and 3-day and 7-day NMDA). When analysed separately for the middle and peripheral regions, the differences were similar to the overall averaged counts (Fig. 2.10; $P < 0.05$).

Figure 2.13 shows immunohistochemical labeling of calretinin in retina from control eyes and NMDA-treated eyes after 8 hour, 24 hour, 3 day and 7 day treatment. Histology shows calretinin-positive cells in both ganglion cell layer and inner nuclear layer, suggesting staining of both RGCs and amacrine cells.

Comparison between RGC markers

Comparison between RGC markers at different time points demonstrated no statistically significant differences between any of the labels ($P > 0.1$). When stratified according to location (central, mid, periphery), there were similarly no statistically significant differences between any of the RGC markers ($P > 0.1$).

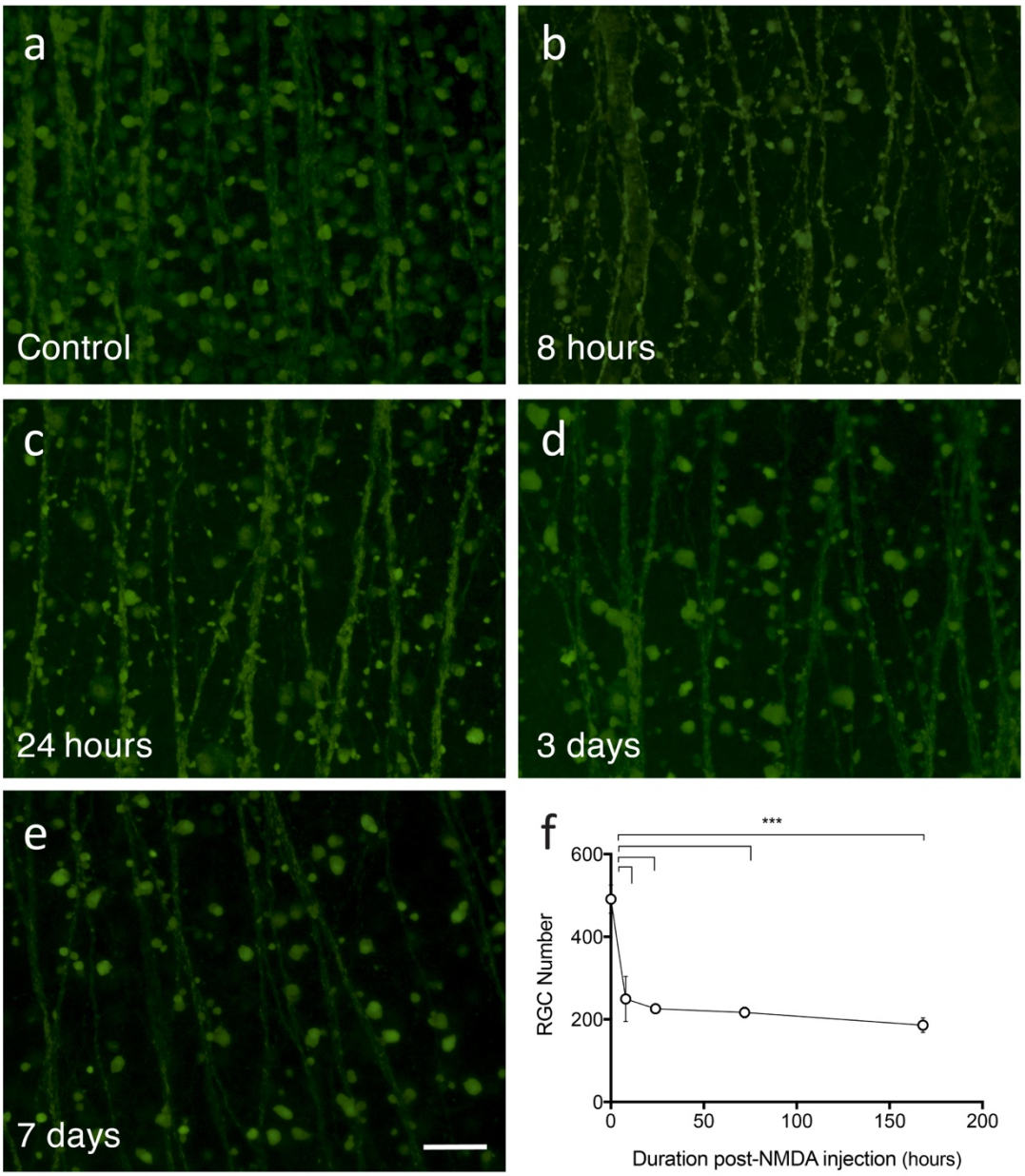

Figure 2.9. (a – e) Photographic representations of γ-synuclein-positive cells at all time points (Control, 8 hours, 24 hours, 3 days and 7 days) and (f) graphical summary of averaged raw counts of γ-synuclein -positive cells over time after NMDA exposure (n = 5-7). *P < 0.05, **P < 0.01, ***P < 0.001 by one-way ANOVA test followed by a Tukey multiple-comparison test. Scale bar = 50 μm. *RGC*, retinal ganglion cell.

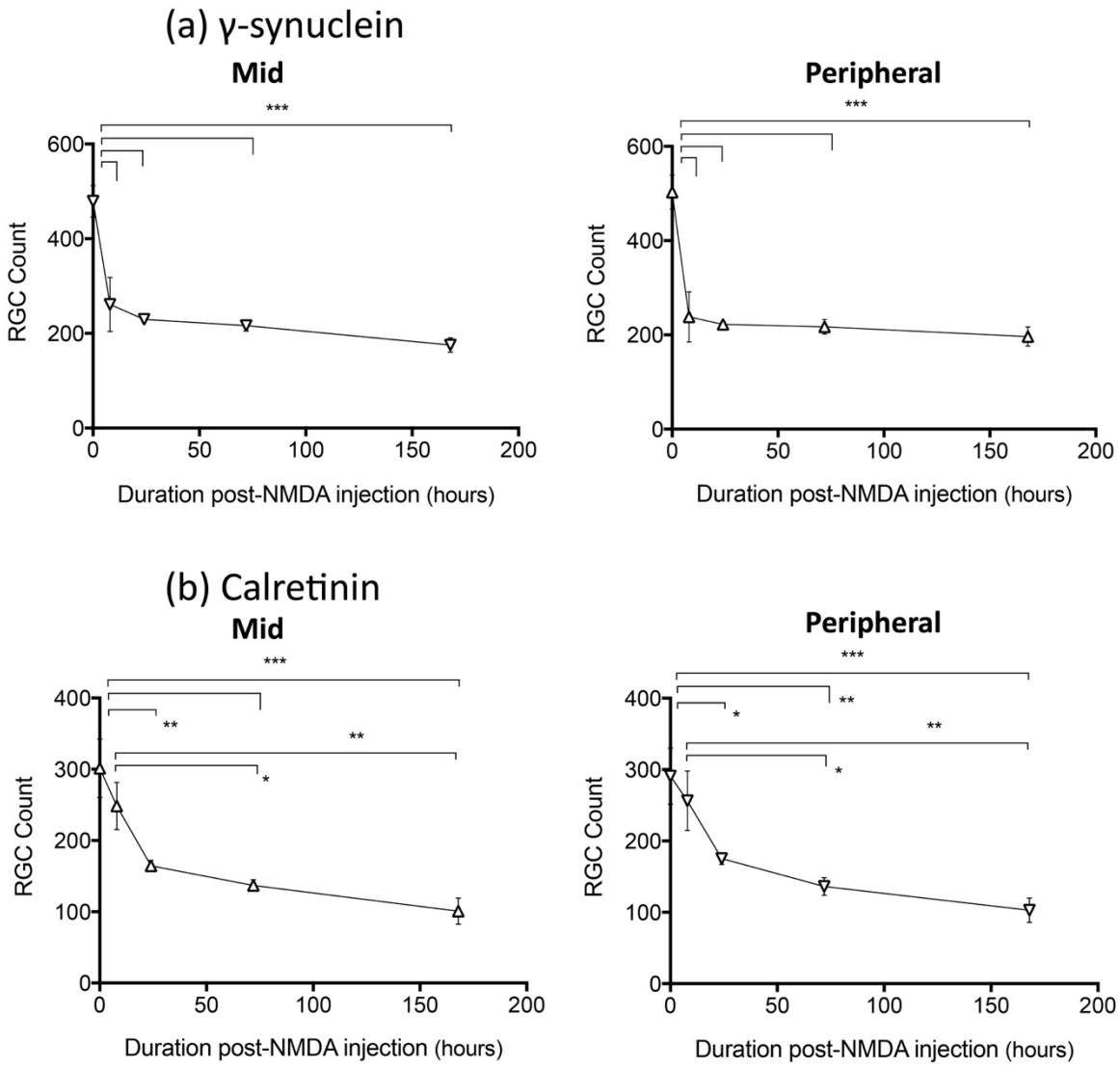

Figure 2.10. Averaged cell numbers for mid and peripheral areas of the retina in (a) γ-synuclein-positive and (b) calretinin-positive cells over time after NMDA exposure (n = 5-7). *P < 0.05, **P < 0.01, ***P < 0.001 by one-way ANOVA test followed by a Tukey multiple-comparison test. *RGC*, retinal ganglion cell.

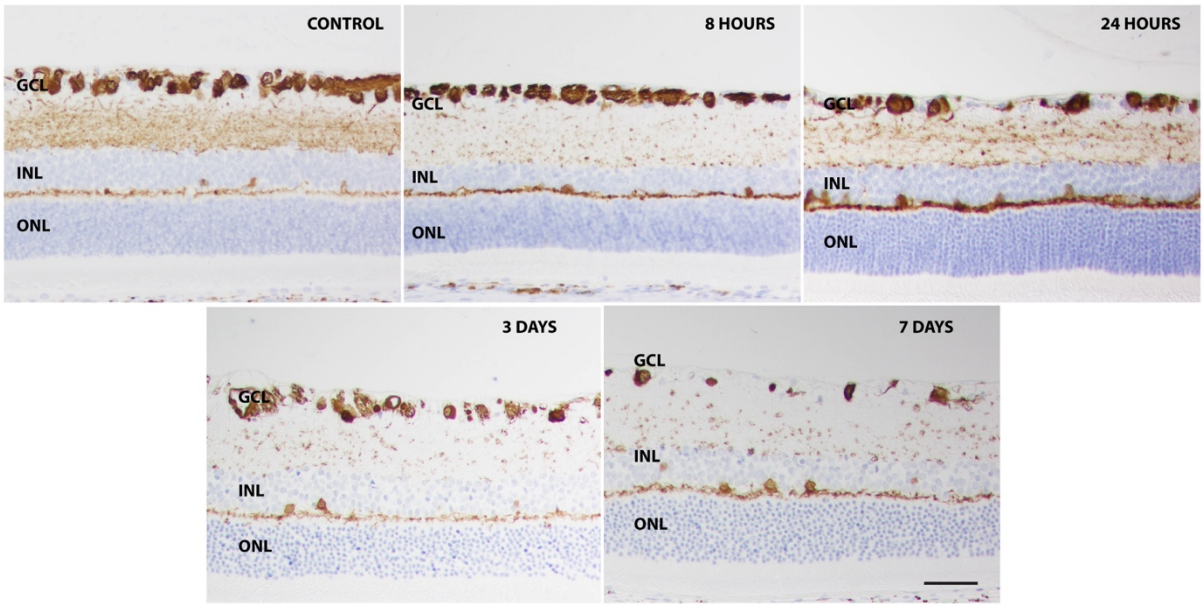

Figure 2.11. Histological cross-section of central retina in eyes treated with NMDA injection. Retinal staining with anti-γ-synuclein showing progressive loss of retinal ganglion cells for control eyes and eyes harvested at 8 hours, 24 hours, 3 days and 7 days after NMDA injection. Scale bar = 100 µm. *GCL*, ganglion cell layer; *INL*, inner nuclear layer; *ONL*, outer nuclear layer.

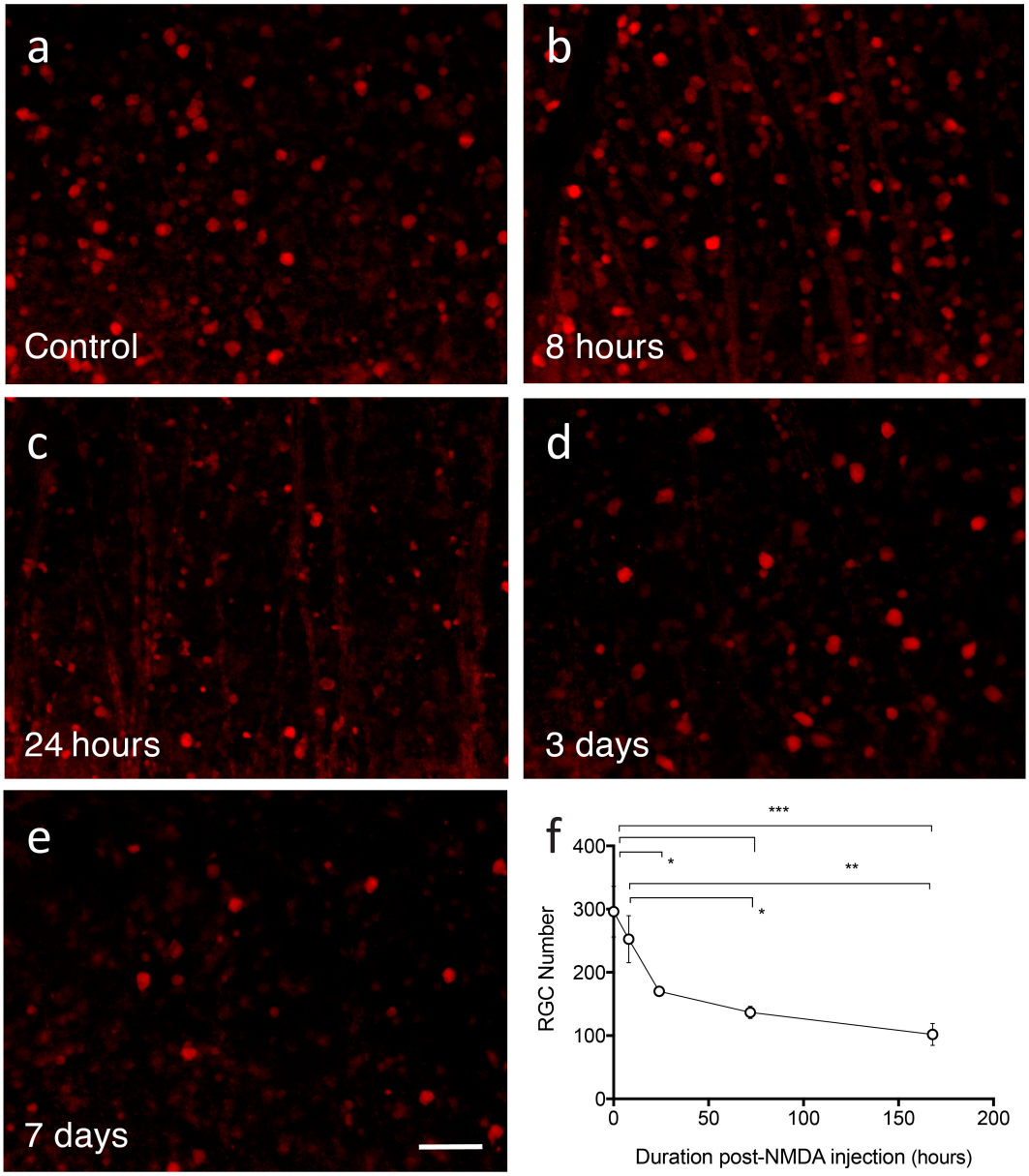

Figure 2.12. (a – e) Representative images of calretinin-positive cells in untreated retinas (a) and retinas exposed to NMDA for (b) 8 hours, (c) 24 hours, (d) 3 days and (e) 7 days. (f) Plot quantifying the effect of NMDA on calretinin-positive cells over time after NMDA exposure (n = 5-6). *P < 0.05, **P < 0.01, ***P < 0.001 by one-way ANOVA test followed by a Tukey multiple-comparison test. Scale bar = 50 µm. *RGC*, retinal ganglion cell.

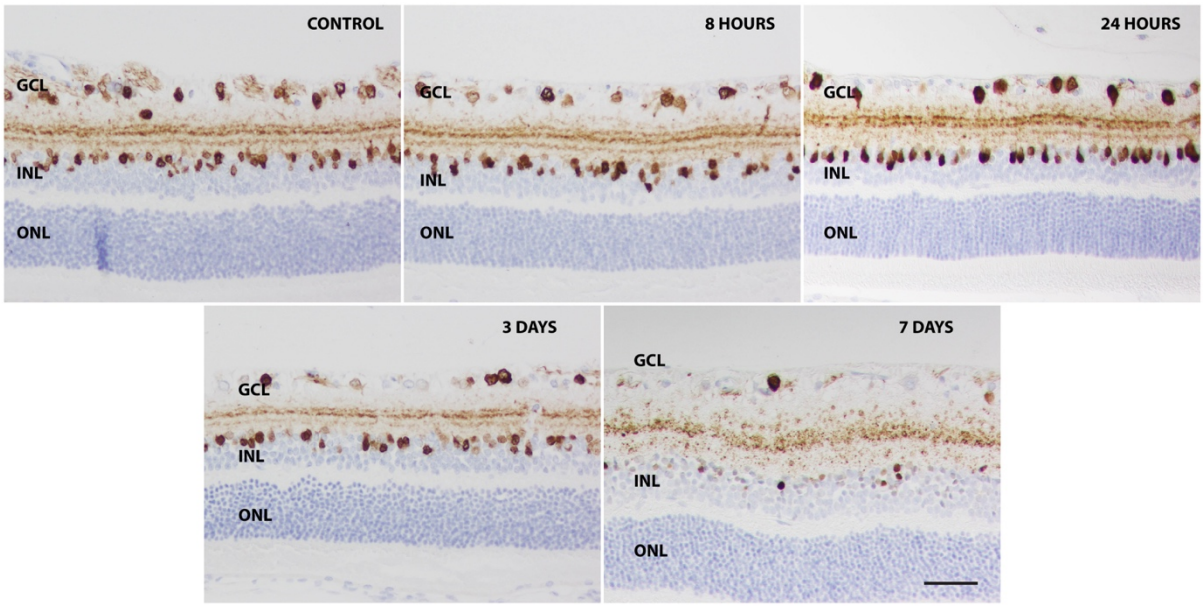

Figure 2.13. Histological cross-section of central retina in eyes treated with NMDA injection. Retinal staining with anti-calretinin showing progressive loss of retinal ganglion cells for control eyes and eyes harvested at 8 hours, 24 hours, 3 days and 7 days after NMDA injection. Scale bar = 100 µm. *GCL*, ganglion cell layer; *INL*, inner nuclear layer; *ONL*, outer nuclear layer.

2.4 Discussion

In this study, the temporal profile of RGC loss using four distinct RGC markers was examined in a well-described neurotoxicity model. NMDA-induced excitotoxicity represents an RGC injury model that is widely used for the study of retinal neurodegeneration. NMDA is a non-metabolisable analogue of the major excitatory retinal neurotransmitter, glutamate. The application of NMDA to a nervous tissue will therefore persistently activate systems designed to be excited under normal circumstances by glutamate; it will therefore "excite a neuron to death," hence the term excitotoxicity. NMDA stimulates a specific class of ionotropic glutamate receptors, the NMDA-type receptors, leading to opening of ion channels which are selective for calcium and sodium, and, to a lesser extent, potassium. Cation entry after NMDA receptor stimulation activates a wide range of cellular processes including opening of intracellular calcium stores, activation of proteases, mitochondrial dysfunction, osmotic shock, energy depletion and ultimately, cell death, either by necrosis – often accompanied by cell lysis - or apoptosis. Within the retina, NMDA-type glutamate receptors are predominantly expressed on RGCs and this explains why these neurons are particularly susceptible to NMDA-induced excitotoxicity.

In attempting to measure RGC death, even in a relatively specific model such as NMDA toxicity to the retina, it is necessary to define what we mean by the term "cell death". It is both difficult to define at what point in the demise of a cell that "death" will occur and difficult to show, in practical terms, that this point has occurred. In terms of the former, it can be stated that a cell is no longer able to survive when central cellular functions are irretrievably lost; therefore, it can presumably also be stated that when a cell has passed such a point, it is dead. How do we detect when a cell has passed this point? We can assay for a specific function, such as cessation of production of a specific or key cellular mRNA species.[115] However, it can be argued that such an action is induced quickly in stressed RGCs and is either not directly associated with the mechanics of cell death or represents a reversible process.[116,130] Either way, this is not, hence, a true measure of cell death. We believe

that a better way to detect RGC death is to follow temporal changes in expression of protein markers, which are specific to these cells in their live and normally functioning state, in situations when we know these cells are damaged or lost. The issue here is that we do not know how a particular antigen will respond to injury and whether this response will define a loss of function, or loss of a particular cell or whether it will follow an independent time course or remain unaffected. We took this rationale into the present study: we induced RGC death in a manner which is well described, such that we know the temporal profile of RGC loss, and examined how four functionally different antigenic markers of these cells were affected. These data were compared and then related to published information regarding the time course of RGC death after retinal NMDA treatment.

In this study, four different markers for RGCs were compared: Brn3a, γ-synuclein, calretinin and RBPMS. The different RGC antigen markers examined represent proteins with very different functions. It was interesting to determine whether these markers responded differently to injury because of their diverse functioning, or in a similar way to the insult because they are all expressed by RGCs. Of the protein markers analysed, Brn3a is a transcription factor, a member of POU4f transcription family, of which its expression levels likely reflect the physiological status of RGC. RBPMS is a RNA-binding protein, a member of the RNA recognition motif family, involved in the regulation of gene expression at the post-transcriptional level, including pre-mRNA-processing, RNA stability, transport, localization and translational regulation. γ-synuclein and calretinin are cytoplasmic proteins that reflect more the actual physiology of the cell.[23,28] γ-synuclein is a small natively unfolded protein localized adjacent to the RGC nuclear marker Brn3a at the perinuclear area as well as in the axons.[25] Calretinin, on the other hand, is a cytoplasmic calcium-binding protein likely involved in control and modulation of functioning, buffering, transport and regulation of this cation in signal transduction processes.[121,131,132]

The use of immunofluorescence on whole-mounts as a means of quantification to describe the temporal characterisation of RGC loss after NMDA-induced retinal excitotoxicity is relatively new. Some of the markers used in the present study have, however, been used to label RGCs after optic nerve crush and optic nerve transection; both models of RGC loss via apoptosis,[23,28,29,120,133] the former engendering a slower loss of these cells.[29,134] Significant RGC loss, as defined by labelling for Brn3a and RBPMS on retinal whole-mounts, has, for example, been demonstrated at only 5 days after optic nerve transection, and this occurred for a further 2-3 weeks.[23,28,29,133,134] In other related optic nerve crush models, up to 80% of both Brn3a and RPBMS were shown to be significantly reduced at 7 – 9 days after the nerve injury.[28,135,136]

Kwong and colleagues have demonstrated that the greatest occurrence of apoptotic RGC death after NMDA administration to the rat retina, as detected by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL), was noted at 24 hours after NMDA administration.[124] Our study aimed to compare how the rates of decline of our four selected RGC markers compared with the data of Kwong and colleagues.[124] We showed that indeed in three markers – Brn3a, RBPMS and γ-synuclein – the rate of cell loss was maximal between 8 and 24 hours after NMDA administration. Calretinin-positive cells, on the other hand, exhibited a slower steady decline throughout the 7 days of observation. It is important to note, here, however, that calretinin is also expressed by some displaced amacrine cells that are situated in the ganglion cell layer; this would clearly affect the observed data if these cells were unaffected by NMDA, which is known to be the case.[137] In this case, there would be a certain percentage (i.e. the displaced amacrine cells) that would not be lost and it would be expected that the counts would plateau at a value reflective of the percentage of cells which were not RGCs and therefore not affected by NMDA. Calretinin could also be a useful marker of RGC loss if susceptible displaced amacrine cells were lost at the same rate as RGCs such that cell loss resembled that of a single population. Despite the approximate agreement in detection of time of maximum rate of cells loss for each of the markers, we have also shown that they described

variation in their overall temporal profiles for RGC loss. Brn3a and RBPMS displayed similar trends, i.e. a steep reduction in up to 79% cell loss within the first 24 hours, followed by minimal drop throughout the rest of the experiment, which is consistent with the data of Kwong and colleagues, showing that maximal apoptosis occurs within the first 24 hours of NMDA exposure and then tails off after this.[124]

Labelling for both Brn3a and RBPMS indicated similar overall loss of about 85% of immunoreactivity by the end of our experiment (7 days). This figure is slightly greater than that reported in the literature for NMDA-induced RGC loss (about 80%) quantified via histological and retrograde labelling methods.[128,129] In optic nerve crush and transection models, both Brn3a and RBPMS are reduced to 15-20% at 7-9 days post-injury.[28,136] In retinal ischaemia models via high intraocular pressure, level of injury is correlated to the duration of ischaemia, although an 80% of cell loss (retrograde labelling) have been noted after 7 days of 120-minute ischaemia.[138]

Calretinin-positive cells in our study, on the other hand, had a slower rate of decline with an overall loss of 65% at 7 days. The lower overall loss of this antigen is likely due to the fact that calretinin is also expressed in all displaced amacrine cells, where a subset of the population is sensitive to NMDA-induced damage.[139] Siliprandi and colleagues noted that this subset of amacrine cells are likely cholinergic amacrine cells, which are well known to also express NMDA receptor subunit NR1.[123,128] In our study, we included cells with soma size of 10µm or higher in diameter in attempt to exclude displaced amacrine cells. However, soma size of displaced amacrine cells in the ganglion cell layer ranges from 7 – 12µm, hence some would be inevitably included in our quantification.[139] In addition, the diameter size of RGCs range from 5 – 33µm, which means that the smaller RGCs which were not counted would not have been included in analysis.[5,126,140] It is however not known whether the rate of decline in RGCs from NMDA-induced toxicity is also similar in displaced amacrine cells; if not, this would explain the difference in rate of decline in calretinin-positive cells compared to Brn3a-, RBPMS-, and γ-synuclein-positive cells from NMDA toxicity.

γ-synuclein is a protein with an unknown function which is expressed throughout RGC cytoplasm and axons. There is suggestion that it may be involved in microtubule regulation of the cell cytoskeleton.[141] Although in human eyes, γ-synuclein was noted to predominantly label RGCs,[25] it has also been observed to stain the inner nuclear layer in rats, suggesting its presence in amacrine cells.[142] In our study, however, γ-synuclein stained cells in the ganglion cell layer as well as a number of cells in the outer region of the inner nuclear layer which resemble horizontal cells. γ-synuclein in the ganglion cell layer was observed to co-label with Brn3a, which accounts for 92 – 96% of FluoroGold-labeled RGCs in rodents.[25,28] As we have also applied similar inclusion criteria as for calretinin, we did not include smaller cells in the ganglion cell layer in our quantification; this, thus, provided us with a lower overall absolute count. We showed that γ-synuclein declined at a similar rate to Brn3a and RBPMS, but with a much lower overall loss with an average of 40% remaining immunoreactivity at 7 days after NMDA treatment. This is about twice the percentage of RGC survival (quantified via histological and retrograde labelling methods) observed from previous NMDA treatment with an equivalent dose.[128,129] This could be due to the antigen being a structural protein which may remain in situ even after cell death. This may be unlikely as NMDA-induced retinal toxicity was shown to cause cell death via apoptosis, in which case it would be expected that there would not be any accumulation of cellular debris, provided that timely functional phagocytosis occurs.[126,143] On the other hand, the end-point of the remaining γ-synuclein in our study may reflect loss of one population cells, as well as new expression in surviving cells. It is unknown whether γ-synuclein is newly expressed in tissues undergoing excitotoxic stress, but γ-synuclein has been shown to be overexpressed in cancer cells, which we know have intrinsic oxidative stress from oncogenic stimulation.[144,145] If this is the case, the level of γ-synuclein may reflect a dynamic equilibrium between cell loss and new antigen expression, which is also in line with previous observation that different subpopulations of RGCs respond to NMDA in a differential manner.[126]

2.5 Conclusion

Quantification of labelling for Brn3a and RBPMS in our model of NMDA-induced excitotoxicity provided most resembled rates of RGC loss that have been reported in previous studies. Both labels had the best signal-to-noise ratios and their proportion of cell loss was similar to each other. Nevertheless, quantification of RGC loss via counting of either of these two antigenic markers may be overestimated due to their being transcription factors and thus not necessarily reflecting true cell loss. On the other hand, γ-synuclein showed a similar rate of cell loss, but with a less marked overall end-point, which may either be an effect of the antigen remaining after cell death or the dynamic equilibrium of cell loss versus new antigen expression in surviving cells. Calretinin did not reflect the rate of cell loss that has been reported and, additionally, is not specific to RGCs. It is, therefore, not appropriate for calretinin to be used for quantification of RGC loss. Amongst all tested antigens, Brn3a and RBPMS are the mostreliable and truly representative antigens expressed by RGCs that are, furthermore, easiest to quantify due to their good signal-to-noise ratio and solely perikaryal labelling.

3 Neuroprotection of the Retina with Creatine

3.1 Introduction

The concept of neuroprotection, namely the mechanisms and strategies aimed at directly preventing neuronal injury or death, has generated an abundance of recent research interest, particularly in the case of the central nervous system (CNS), where damaged neurons do not have the ability to regenerate.[19] It is believed that slowing or halting the actual degenerative process in this manner is a reasonable and attainable goal that could provide treatment for a range of neurological disorders. In the retina, a part of the CNS, multiple types of neurons are present, including retinal ganglion cells (RGCs). RGCs have relatively long and incompletely myelinated axons that extend through the optic nerve to the lateral geniculate nucleus of the brain.[3] Because of the relative length of these axons and the fact that they lack a myelin sheath until they are into the optic nerve, RGCs have an increased vulnerability to any local mechanical, vascular or metabolic perturbation, particularly in the retina or optic nerve head. Thus, damage to or death of these cells is observed in multiple blinding retinal conditions, for example, glaucoma, diabetic retinopathy and ischemic optic neuropathy.[19]

The retinal degenerating condition, glaucoma, has known similarities with neurodegenerative conditions elsewhere in the central nervous system. At the molecular level, these similarities include processes such as oxidative stress, mitochondrial dysfunction, excitotoxicity and Ca^{2+} neurotoxicity. These mechanisms, in particular, are regarded as important in neurodegenerating conditions as they can ultimately lead to cell apoptosis (i.e. programmed cell death).[14,63,146] Stimulation of these mechanisms may be via mechanical or vascular injuries, which manifest as trauma and ischaemia/hypoxia, respectively. Often, the end result of such insults is cellular energy failure, and this, itself, often leads to further oxidative and mitochondrial damage.[4] It would therefore be ideal if there were agents available that could intervene at any of these steps, as these would theoretically slow or abrogate progression of degeneration.

Strategies aimed at enhancing cellular energy metabolism have been attempted with variable success in a variety of neurological disorders. Nicotinamide, which acts as a substrate for Complex I in the respiratory chain, and also acts as a free radical scavenger and an inhibitor of poly-ADP-ribose polymerase, has been shown to attenuate ischaemic and phototoxic injuries to RGCs.[147] Coenzyme Q_{10}, a potent anti-oxidant and a component of the mitochondrial respiratory chain that carries electrons from complexes I and II to complex III, moreover, was shown to be neuroprotective in animal models of Huntington's disease and Parkinson's disease, as well as in a glaucoma model generated by elevating intraocular pressure (IOP).[148-150] It is interesting to also note that short-term hyperglycaemia and intraocular administration of glucose have also been shown to attenuate ischaemic retinal injury.[112] Recently, our laboratory has demonstrated significant RGC protection via subconjunctival glucose administration in a rat model of acute elevated IOP.[68] However, concerns remain regarding the systemic uptake of glucose and whether hyperglycaemic microvascular complications may result from its application.

Creatine, a guanidine compound that acts as an energy buffer as well as an anti-oxidant, and which occurs naturally in vertebrates, has been suggested for use in the treatment of major neurodegenerative disorders. Experimental testing of this compound has shown a degree of neuroprotection in both in vitro and in vivo animal models.[74,91] Although results so far have not supported the presence of any neuroprotective effects in human trials, creatine treatment has nonetheless been proven to be extremely tolerable by patients and so studies persist.[111] To date, there are no studies that have examined the direct neuroprotective efficacy of creatine in situations of retinal neurodegeneration. However, S-adenosyl-L-methionine, which is a biosynthetic precursor of creatine was able to restore photoreceptor function in a rat model of retinal ischaemia.[70] Taking all of these data into account, it was believed that creatine would offer a good chance at providing protection to retinal neurons. This study, therefore, aimed to assess the neuroprotective properties of creatine in

different retinal neurotoxicity paradigms: mixed retinal neurons in culture subject to metabolic compromise, excitotoxicity of RGCs in situ and pan-retinal ischaemia in vivo.

3.2 Methods

Experimental design

There were two phases to the overall study: (1) To assess the effect of creatine in mixed retinal cultures exposed to energetic compromise with the mitochondrial complex IV inhibitor, azide; and (2) to assess putative neuroprotective effects of creatine in rat models of retinal ischaemia and excitotoxicity.

Materials

General cell culture media and reagents, including foetal bovine serum (FBS), were obtained from Invitrogen (Mulgrave, Victoria Australia). Culture vessels (flasks and plates), polypropylene centrifuge tubes of all sizes, and CellPlus charge-coated 24-well plates were from Sarstedt Pty (Adelaide, Australia). All other general chemical reagents, except where noted, were from Sigma-Aldrich Chemical Company (Castle Hill, New South Wales, Australia).

Animals

This study was approved by the Animal Ethics Committees of SA Pathology/Central Adelaide Local Health Network and The University of Adelaide and conformed to both the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2013) and the Association for Research in Vision and Ophthalmology Statement for The Use Of Animals In Ophthalmic And Vision Research. All animals were obtained from the University of Adelaide, South Australia. For culture studies, Sprague-Dawley rat litters (2-4 days post-partum, average of 8-12

pups per litter) were obtained and for the in vivo experiments, adult Sprague-Dawley rats (6-10 weeks) were used.

Culture Model

Rat retinal cell cultures comprising glia, photoreceptors and neurons were prepared from the pups via a trypsin- and mechanical-digest procedure.[151] After tissue dissociation, cells were dispensed onto 13 mm diameter borosilicate glass coverslips coated previously with poly-L-lysine (5 µg/mL, 15 minutes) in 24-well culture plates for immunocytochemical, fluorescent dye-labelling, or apoptotic analyses. Mean cell density at seeding was approximately 0.5 x 10^6 cells/mL. Subsequently, cultures were grown at 37˚C in a humidified incubator with 5% CO_2 in growth medium (MEM containing 10% FBS, 91 mg/L gentamicin sulphate, 2.3 mg/L amphotericin B, and 25 mM glucose).

After 6 days in vitro, medium was changed and the cultures were incubated for 24 hours with either creatine (concentrations of 0.5 mM, 1 mM and 5 mM) or standard medium (control group). Some of both the creatine-treated group and the control group were subjected to concurrent incubation for 24 hours of 1 mM NaN_3 and some were treated for 1-hour with 10 mM NaN_3 (10 mM), before cells were fixed 10% (w/v) neutral-buffered formalin in 0.1M phosphate buffer, pH 7.4 (NBF) for immunocytochemical analysis.

Animal Models of Retinal Ischaemia and Excitotoxicity

In both the retinal ischaemia and N-methyl-D-aspartate (NMDA)-toxicity models, rats were randomly assigned to either treatment group, which received 2% creatine feeds (20g/kg) for 4-6 weeks or a control group, which received standard normal feeds throughout the experiment.

For establishment of retinal ischaemia in rats, the left eye was cannulated with a 32-gauge needle attached to a reservoir containing sterile 0.9% (w/v) NaCl. Elevation of the reservoir to two metres above the subject enabled IOP to be raised

to 120 mmHg, which rendered the retina ischaemic. This was maintained for 75 minutes. All rats were killed at seven days following retinal ischaemia and their eyes removed for RGC quantification.

To establish excitotoxicity, the left eyes of all rats were subjected to intravitreal injections (using a 32-gauge needle) of 10 nmol of NMDA, under observation with a dissecting microscope; seven days following NMDA exposure all rats were killed and their retinae harvested for RGC quantification. To assess the effect of creatine on neuronal apoptosis, a separate group of NMDA-treated animals were killed at 8 hours following injection and their retinae processed accordingly. The right eye in each animal was used as an intra-group control.

Electroretinograms for retinal ischaemia model

Rats assigned to the retinal ischaemia model had electroretinogram studies performed in both injured and control eyes before ischaemia (baseline) and three and seven days after reperfusion. Rats were dark-adapted overnight and anaesthetized briefly with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Pupils were dilated with 1% tropicamide. Eyes were anaesthetised with 0.04% oxybuprocaine hydrochloride. *Poly Gel*® lubricating *eye* gel was applied to the cornea before applying corneal electrode, reference electrode (connected to the ipsilateral ear) and ground electrode (on midline dorsal cephalad). Electroretinograms were recorded under general anaesthesia and all experimental preparations were performed under dim red light. Light stimulus (10 µsec; 1 per sec) was provided by a stroboscopic flash placed 15 cm in front of the rat with a 0.75 log-flash intensity ($cd \cdot s/m^2$). Signals were recorded and amplified (gain:1000) by a DC amplifier. The a-wave amplitude was measured from pre-stimulus potential to its trough and the b-wave amplitude from the a-wave trough to the b-wave peak. Ten consecutive responses were recorded and averaged for each animal (n = 7-8).

Immunocytochemistry and Immunohistochemistry

Cells in retinal cultures were fixed with NBF containing 1% methanol for 15 minutes and then washed in standard phosphate buffered saline (PBS). Cells were permeabilized with PBS containing 0.1% Triton X-100 (PBST-0.1%), followed by further washing in PBS and then blocking in PBS containing 3% normal horse serum (PBS-HS). Anti-calretinin (Molecular Probes; mouse) and anti-GABA (Molecular Probes; rabbit) antibodies, diluted in PBS-HS at concentrations of 1:1000 each, were applied overnight at room temperature, after which, coverslips were washed in PBS and labelled by consecutive incubations with appropriate biotinylated secondary antibodies (Vector Laboratories, Abacus ALS, Brisbane, Australia; 1:250 in PBS-HS; 30 minutes) and streptavidin-AlexaFluor 488 or streptavidin-AlexaFluor 594 (Molecular Probes, Invitrogen; 1:500 in PBS-HS; 1 hour). Finally, cells on coverslips were mounted using anti-fade mounting medium (DAKO, Botany, New South Wales, Australia).

For in vivo experiments, all rats were killed by transcardial perfusion with physiological saline and eyes were dissected and fixed with NBF for 24 hours at room temperature. For tissue sectioning, whole eyes with optic nerve attached were processed for routine paraffin-embedded sections. Eyes were embedded sagitally and 5µm serial sections were cut. The cut sections were then deparaffinised, rinsed in 100% ethanol and treated for 30 minutes with 0.5% H_2O_2 to block endogenous peroxidase activity. Antigen retrieval was achieved by microwaving the sections in 10mM sodium citrate buffer (10mM tri-sodium citrate dihydrate in H_2O; pH 6.0). Tissue sections were then blocked in PBS-HS, incubated overnight at room temperature in anti-Brn3a (1:3000 dilution in PBS-HS) and anti-γ-synuclein (1:1000 dilution in PBS-HS) primary antibodies, followed by consecutive incubations with biotinylated secondary antibody and streptavidin-peroxidase conjugate. Colour development was achieved using 3',3'-diaminobenzidine plus H2O2. Sections were counterstained with haematoxylin, dehydrated and mounted. Specificity of antibody staining was confirmed by incubating adjacent sections in the absence of primary antibody.

For whole-mount immunochemistry, posterior eye cups were dissected, permeabilized with PBS containing 1% triton X-100 (PBST-1%), blocked in PBST-1% containing 3% normal horse serum, then incubated in the same solution additionally containing anti-Brn3a (1:600 dilution) and anti-γ-synuclein (1:100 dilution) primary antibodies, overnight at 4˚C for co-labelling. After washing with PBS on the following day, whole-mounts were incubated with secondary anti-goat Alexa Fluor 594 conjugate and anti-mouse Alexa Fluor 488 conjugate for 3 hours at room temperature. Lastly, whole-mounts were rinsed in PBS and mounted using anti-fade mounting medium (DAKO).

TUNEL Staining

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labelling (TUNEL) staining was performed according to previously described methods for cultures and whole-mounts.[152] For cultures, cells on coverslips were fixed as for immunocytochemistry, permeabilised in PBST-0.1% for 15 minutes and washed in PBS. Following this the transferase reaction was performed by incubating coverslips in TdT buffer (30mM Tris-HCl, pH7.2, containing 140mM sodium cacodylate and 1mM cobalt chloride) for 60 minutes at 37^{o}C, with added 0.25U/µl and 10µM biotin-16-dUTP. Reaction was stopped with saline sodium citrate buffer (300mM NaCl, 30mM sodium citrate) for 2 x 15 minutes before blocking reaction with PBS-HS. Labelling was visualised using streptavidin-Alexa Fluor 594. For retinal whole-mount staining, eyes were fixed with NBF as for immunohistochemistry, retina whole-mounted and washed in PBS. The retinal whole-mounts were then permeabilised with PBS containing 3% triton X-100 for 30 minutes, treated with proteinase K (Sigma Aldrich, 10 µg/mL in PBS) for 10 minutes at 37˚C and blocked in PBST-1% containing 3% normal horse serum. The transferase reaction was performed by incubating retinal whole-mounts in TdT buffer overnight at room temperature, with added 0.5U/µl and 10µM biotin-16-dUTP. Reaction was stopped with saline sodium citrate buffer for 2 x 15 minutes. For visualisation, the whole-mounts were incubated with streptavidin-

Alexa Fluor 594 or 488 (1:500) for 3 hours before being mounted using anti-fade mounting medium.

Assessment of Cellular levels of Peroxide in Culture

For determination of reactive oxygen species (ROS) levels in cultures, the ROS-Glo™ H_2O_2 Assay Kit (Promega) was used as per manufacturer's protocol. Briefly, incubations of cells with creatine pre-treatment (24 hours of 5 mM creatine) and controls (standard medium) were performed as described above in our culture model except that cultures were also incubated with H_2O_2 substrates simultaneously with two concentrations of sodium azide (0.1 mM and 1 mM) for one hour. H_2O_2 substrates react with H_2O_2 in the media to produce luciferin precursors. The medium in the 24-well plates was then transferred to 96-well plates and mixed with an equal amount of ROS-Glo™ Detection Solution (D-cysteine and luciferase; 50µL each) for 20 minutes to produce luciferin molecules that react with luciferase to generate a luminescent signal which is proportional to the H_2O_2 concentration. Luminescence was quantified by luminescence spectroscopy using a luminometer (Fluostar Optima; BMG Labtech, Mornington, Victoria, Australia). For quantification, luminescence readings (expressed as luminescence units) from 3 individual coverslips per data point were averaged.

Assessment of Cellular ATP Content

For determination of ATP levels in culture, a firefly bioluminescence assay kit from Sigma-Aldrich Chemical Company was used. Cell samples were obtained by removing culture medium and extracting cellular contents, including ATP, into hot (65˚C) distilled water for 5 minutes to denature ATP-metabolizing enyzmes. ATP levels were then determined in comparison with a standard curve using a luciferin-luciferase assay on a luminometer (Fluostar Optima).

Quantification of Retinal Neurons

To quantify immunocytochemistry in cultures, cells were counted manually in 5 random fields per coverslip and on 6-8 distinct coverslips. Counts were expressed as percentages of non-treated (without NaN_3) control numbers. Similarly for retinal whole mounts, in each of the 4 quadrants, three rectangular areas (central, mid and peripheral) each at 1.2 mm, 1.9 mm and 2.6 mm from the optic disc were analysed, yielding 12 separate retinal areas for RGC counting. Each rectangular area measured 1.40 mm x 1.08 mm. Similarly, counts for whole mounts were expressed as percentages of untreated control numbers (right eye of each animal). Quantification was performed by observers who were masked to the group allocation (creatine vs control).

Statistical Analysis

Culture data were analysed for significance using a one-way ANOVA followed by a Tukey multiple-comparison test. Data from whole mounts were analysed using Student's t-test. Data were expressed as either absolute cell counts or mean percentage of control value $\pm$ SEM. A P value of < 0.05 was considered significant.

3.3 Results

Energetic compromise in culture, as well as NMDA-induced excitotoxicity and high intraocular pressure-induced retinal ischaemia in vivo, all provided consistent and quantifiable level of neuronal death and therefore allowed for dissemination of the possibility of creatine acting as a neuroprotectant.

Neuroprotection by creatine in vitro

Incubation of rat retinal culture with creatine across different concentrations (0.5, 1.0 and 5.0 mM) did not result in significant differences in calretinin- and GABA-positive cell counts (Figure 3.1; $P > 0.05$). In the acute paradigm of metabolic damage, treatment with 10 mM sodium azide for 1 hour or 1 mM sodium azide for up to 24 hours led to significant loss of calretinin-positive and GABA-positive neurons in the rat retinal cultures (Figure 3.2). This was measured by quantifying changes in neuronal labelling as detailed in the Methods section. When treated with sodium azide, loss of GABA-positive neurons was greater, relative to calretinin-positive neurons. Pre-treatment with creatine led to significantly increased calretinin-positive counts across all creatine concentrations (Figure 3.2; $P < 0.01$, $n = 12$). For GABA-positive neurons, only pre-treatment with 5.0 mM of creatine resulted in significantly higher counts compared to the non-creatine controls (Figure 3.2; $P < 0.01$, $n = 13$).

In the chronic paradigm of metabolic damage, treatment with 1 mM sodium azide for 24 hours also led to significant loss of calretinin-positive and GABA-positive neurons. Similar to the acute paradigm, loss of GABA-positive neurons was greater, relative to calretinin-positive neurons. Pre-treatment with creatine led to a significant increased calretinin-positive counts across all creatine concentrations (Figure 3.3; $P < 0.05$, $n = 3$). For GABA-positive neurons, only 5.0 mM of creatine resulted in significantly increased counts (Figure 3.3; $P < 0.05$, $n = 4$).

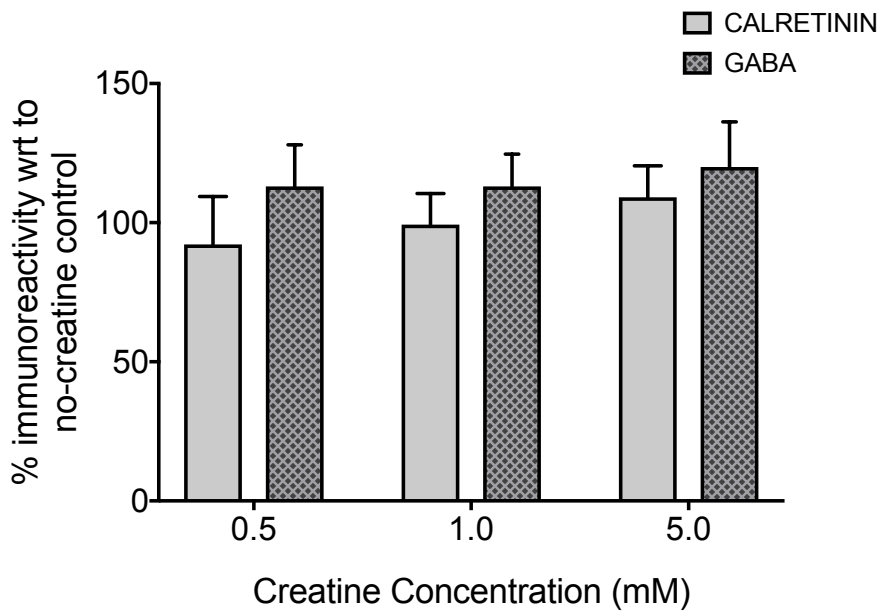

Figure 3.1. Effect of 24 hours of creatine (0.5, 1.0 and 5.0 mM) pre-treatment on calretinin-immunoreactive and GABA-immunoreactive cells in rat retinal cultures, expressed as percentage immunoreactivity compared to no-creatine controls. No significant differences were observed in cell counts between different concentration groups, including control group (one-way ANOVA test followed by Tukey multiple-comparison test, n = 13-14).

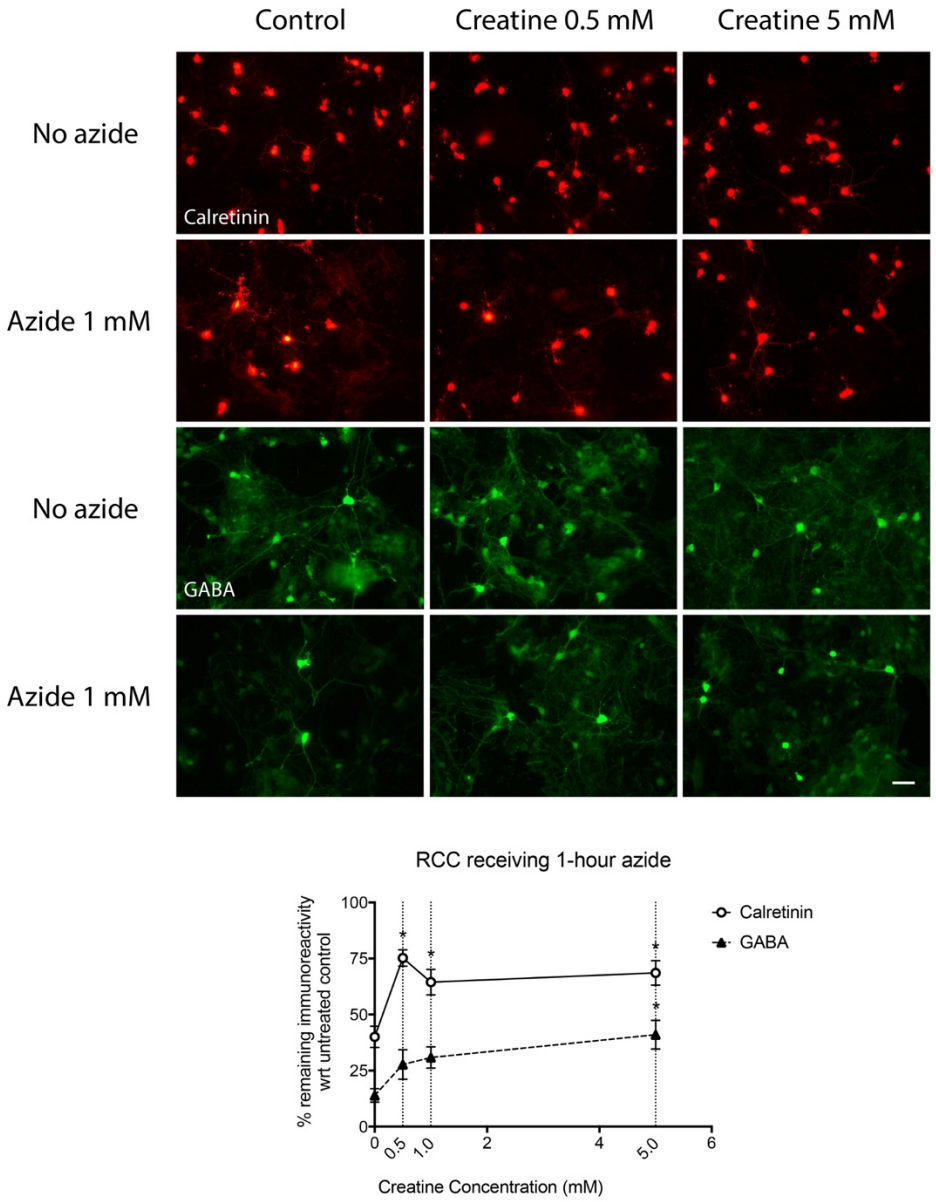

Figure 3.2. Acute metabolic insult to retinal neurons by treating with sodium azide (10 mM) for 1 hour. Significant calretinin- and GABA-positive cell loss was detected in the sodium azide-treated group. Partial protection by pre-treating neurons with creatine for 24 hours was observed for calretinin-immunoreactive neurons (0.5, 1.0, 5.0 mM creatine) and GABA-immunoreactive neurons (5.0 mM creatine). No significant difference was detected in neuron survival after treatment with different creatine concentrations in the absence of sodium azide. *P < 0.01 by one-way ANOVA test followed by Tukey multiple-comparison test (n = 12-13). Scale bar = 20 µM. *RCC*, retinal cell culture.

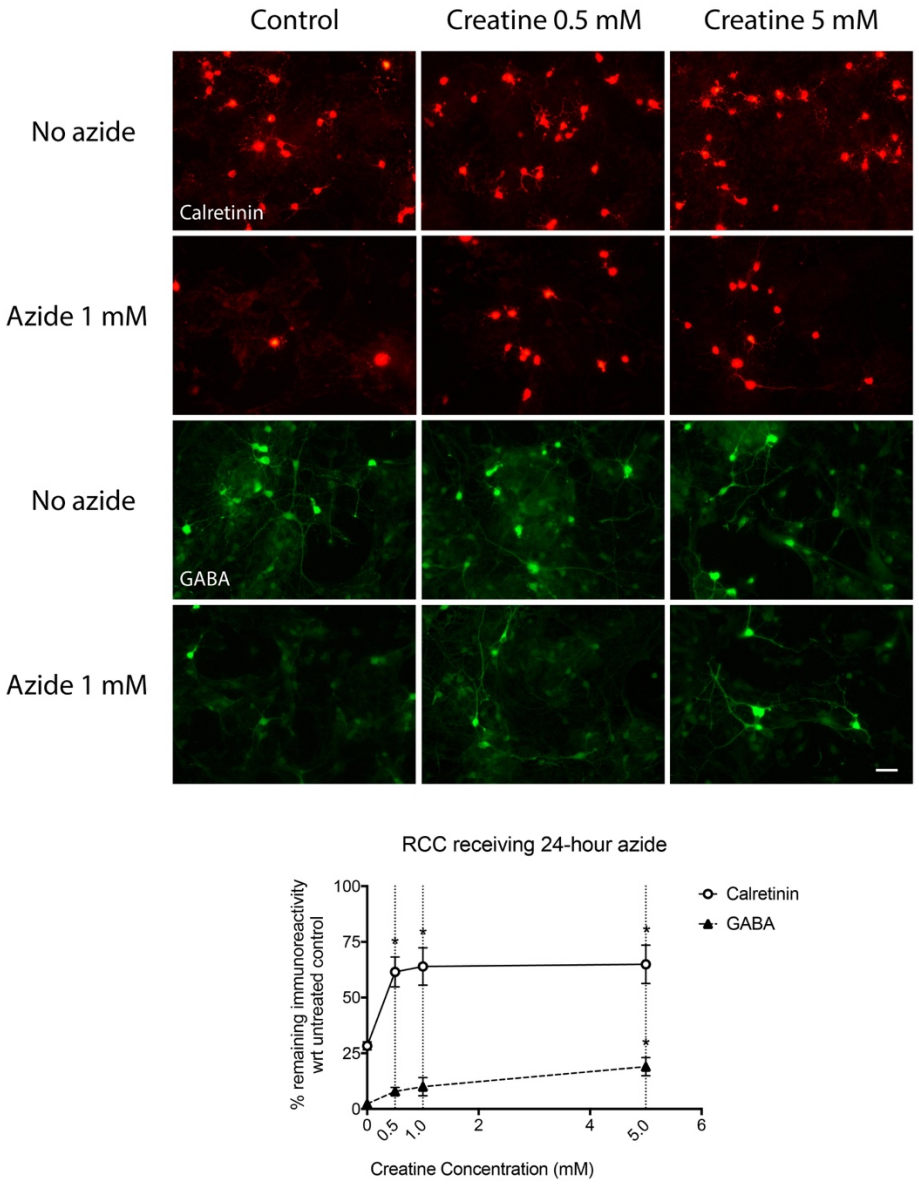

Figure 3.3. Chronic metabolic insult to retinal neurons by treating with sodium azide (1 mM) for 24 hour. Significant calretinin- and GABA-positive cell loss was detected in the sodium azide-treated group. Partial protection by pre-treating neurons with creatine for 24 hours was observed for calretinin-immunoreactive neurons (0.5, 1.0, 5.0 mM creatine) and GABA-immunoreactive neurons (5.0 mM creatine). No significant difference was detected in neuron survival after treatment with different creatine concentrations in the absence of sodium azide. *P < 0.01 by one-way ANOVA test followed by Tukey multiple-comparison test (n = 4). Scale bar = 20 µM. *RCC*, retinal cell culture.

Sodium azide-induced apoptosis

Treatment for 24 hours with 1 mM sodium azide induced an increase in detectable TUNEL-positive cell nuclei in retinal cultures (Figure 3.4). Concurrent incubation with creatine gave rise to a reduction in TUNEL-positive cell nuclei, although this was only significant in the 5 mM creatine group (58% reduction; Figure 3.4, $P < 0.05$, $n = 4$).

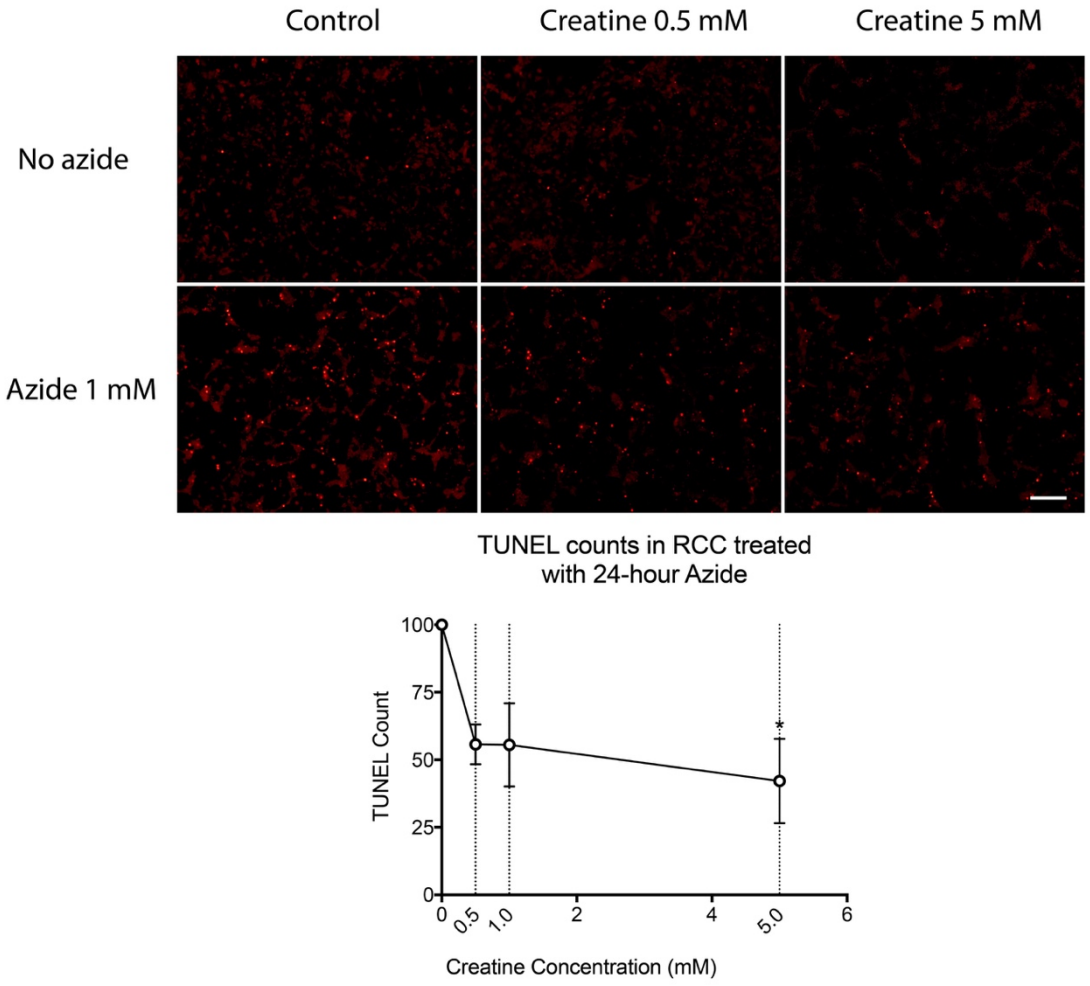

Figure 3.4. Sodium azide (1 mM) 24-hour treatment in rat retinal cultures led to increased TUNEL labelling. Concurrent incubations with creatine (0.5 mM and 5 mM shown) resulted in reduced apoptosis compared to sodium azide alone (photomicrographs and graph). *$P < 0.05$ by one-way ANOVA test followed by Tukey multiple-comparison test ($n = 4$). Scale bar = 100 µM. *RCC*, retinal cell culture.

Effect of creatine on reactive oxygen species (ROS)

Incubation with sodium azide (0.1 mM and 1.0mM) for one hour produced mild rises in ROS levels compared to vehicle-treated controls (Figure 3.5; 107 ± 14 % and 118 ± 4 %, respectively; n = 3). Pre-treatment with 5mM creatine for 24 hours resulted in a significant reduction in azide-stimulated ROS levels (Figure 3.5; 0.1mM azide: 89.3%, P = 0.30; 1.0mM azide: 84.9%, P = 0.007; n = 3).

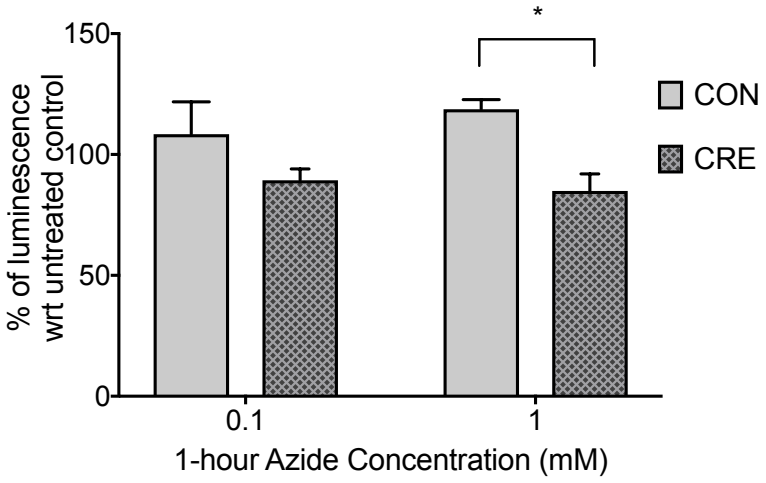

Figure 3.5. Level of H_2O_2 in culture expressed as percentage of luminescence in 1-hour azide-treated cultures compared to controls (no azide) with and without creatine pre-treatment. *P < 0.01 by one-way ANOVA test; n = 3.

Effect of creatine on ATP levels

Figure 3.6 shows the level of ATP in cultures treated with sodium azide (10 mM) for 1 hour. The effect of creatine pre-treatment is also shown (5 mM for 24 hours). Pre-treatment with creatine had no significant effect on azide-reduction in ATP content of cultures (P = 0.59; n = 3).

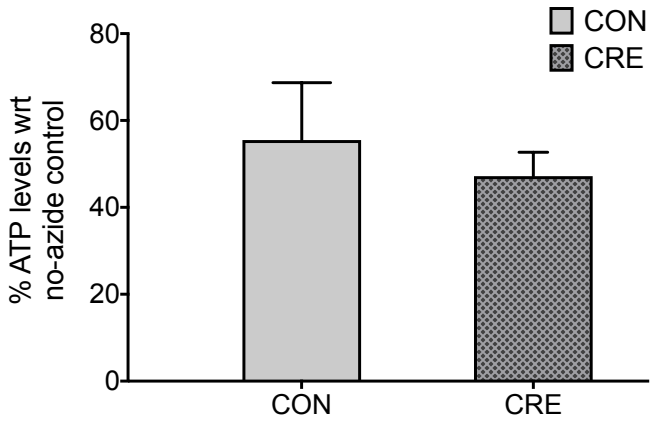

Figure 3.6. ATP content of cultures relative to levels in untreated controls after 1-hour treatment with sodium azide (10 mM). Also shown is the effect of pre-treatment for 24 hours with 5 mM creatine. No significant difference was noted when comparing creatine plus sodium azide-treated with sodium azide alone-treated ($P = 0.59$ by one-way ANOVA test; $n = 3$).

Effect of creatine on RGC loss due to excitotoxic challenge with NMDA

Treatment with NMDA resulted in significant damage to the RGCs after 7 days. The effect of creatine administration on RGC survival from NMDA is shown in Figure 3.7. In NMDA-treated eyes, although there was an observed trend towards RGC neuroprotection from creatine, the differences between controls and creatine-fed animals for both Brn3a-positive and γ-synuclein-positive RGCs were not statistically significant (Figure 3.7; $P > 0.05$; n = 15-18). When distribution of RGCs were stratified according to distance from the optic nerve head (central, middle and peripheral), no significant differences were observed (Figure 3.8).

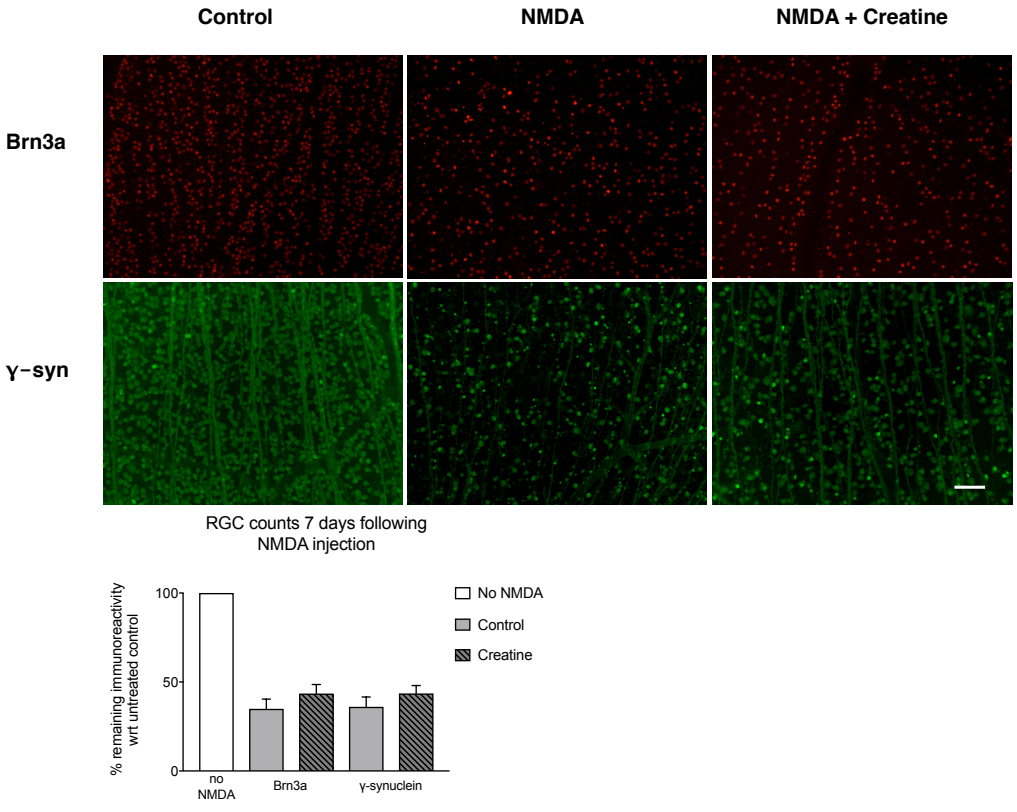

Figure 3.7. Excitotoxic challenge to retinas as induced by intravitreal injections of 10nmol NMDA led to significant losses of Brn3a-positive and γ-synuclein-positive cells. Prophylactic treatment with oral creatine did not result in any significant protection from this cell loss (Student T-test, n = 15-18). Scale bar = 100 µM.

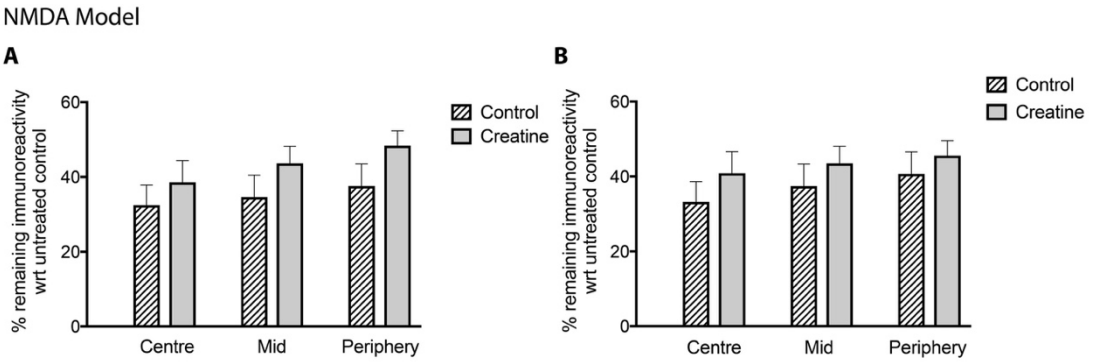

Figure 3.8. Summary of Brn3a- (A) and γ-synuclein-immunoreactive (B) cell loss, stratified based on retinal location into central, middle and peripheral counts, in both control and creatine-treated groups in NMDA-treated eyes. No statistical significant differences were found in any control-creatine comparison groups (Student paired T-test, n = 15-18).

NMDA-induced RGC apoptosis

When animals were killed at 8 hours post-NMDA injection and retinal whole-mount preparations were labelled by the TUNEL reaction alongside anti-Brn3a antibodies, there was an observed reduction in Brn3a-positive RGCs as well as an accompanying increase in labelled apoptotic cells in the retinal ganglion cell layer. The apoptotic cell nuclei detected via the TUNEL reaction corresponded well with shrunken Brn3a-positive cells at the retinal ganglion layer (co-labelling in Figure 3.9). When both TUNEL-positive and Brn3a-positive cells were quantified, no differences were detected when comparing between the control and creatine-fed animals (Figure 3.10; $P>0.05$, n = 8).

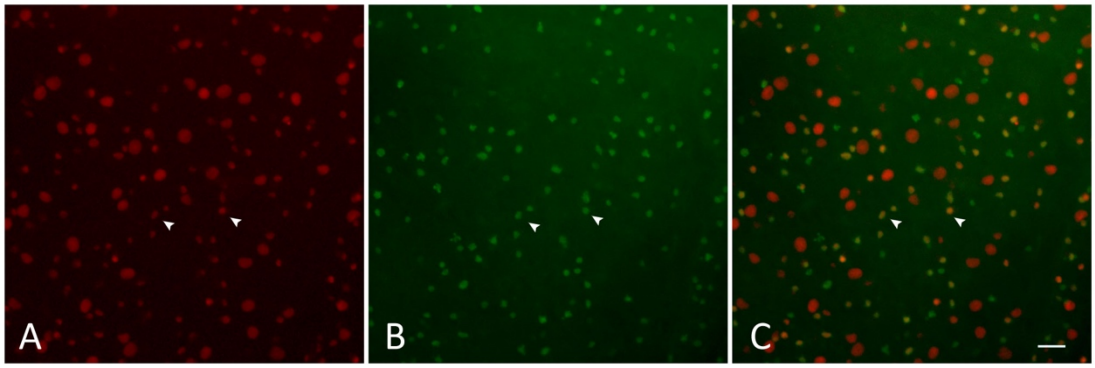

Figure 3.9. Immunofluorescence showing (A) Brn3a (red) and (B) TUNEL (green) labelling of retinal whole-mounts from eyes 8-hours post-NMDA (10 nmol) injection. (C) Co-labelling of both markers shows apoptotic retinal ganglion cell nuclei (arrowheads). Scale bar = 20 µM.

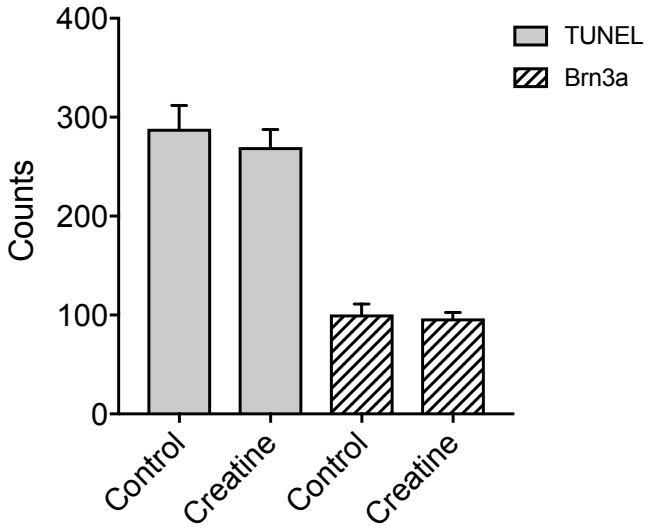

Figure 3.10. Mean TUNEL and Brn3a counts on retina 8 hours after NMDA injection. No statistically significant differences were detected between control and creatine counts in either case (Student T-test; n = 8).

Histological sections of eyes treated with NMDA

Compared to controls, NMDA induced a loss of inner retinal elements showing a loss of cells in the ganglion cell layer (reflected by reduction of staining by Brn3a and γ-synuclein) and mild thinning of the inner plexiform layer. No differences in labelling were detected in eyes pre-treated with creatine (Figure 3.11).

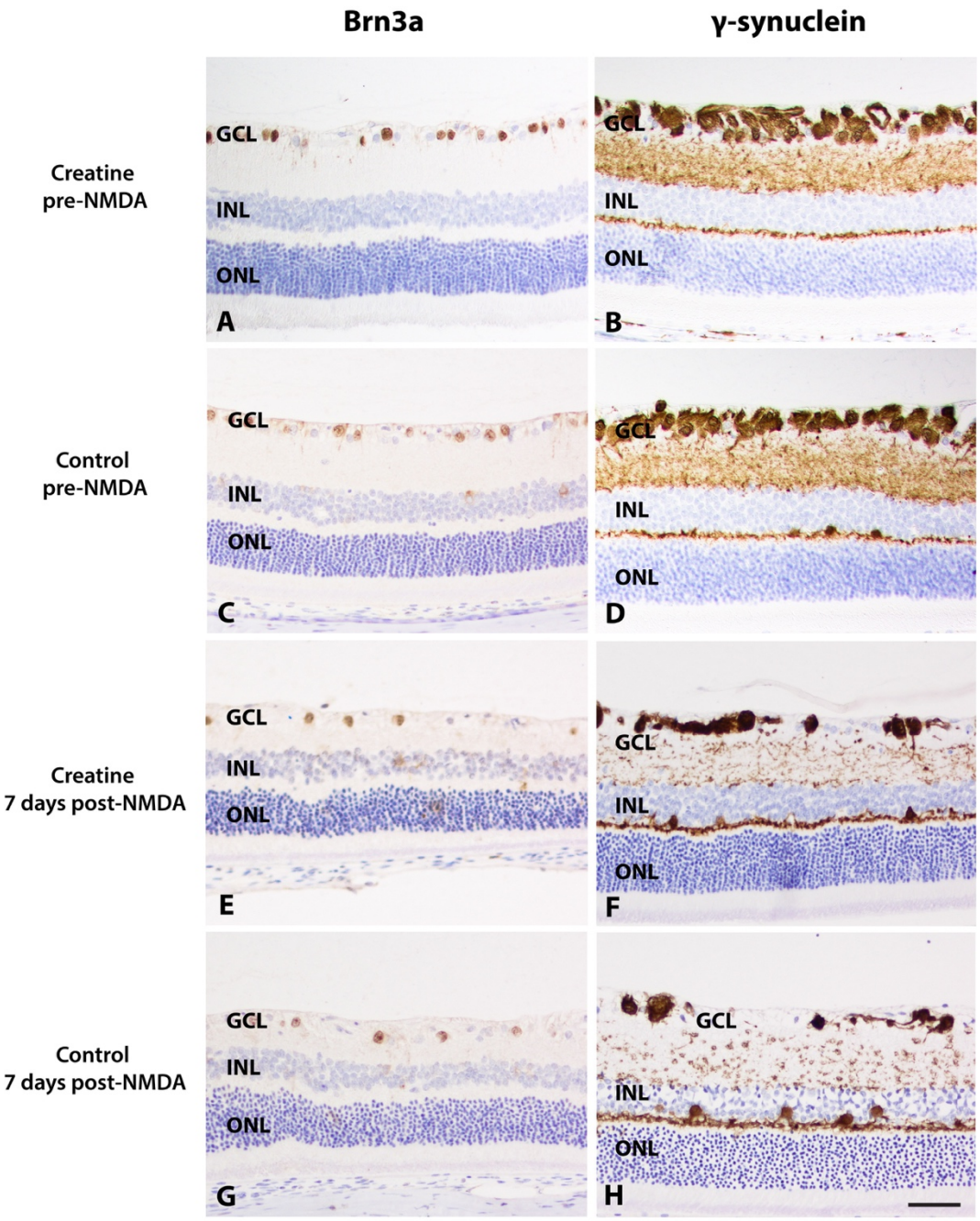

Figure 3.11. Photomicrographs showing retinal histology in tissue cross-sections labelled with Brn3a and γ-synuclein for both control/creatine-treated eyes (A-D) and eyes subjected to intravitreal NMDA (10 nmol) injections, 7-days post-treatment (E-H). Retinal ganglion cells, labelled with Brn3a and γ-synuclein, were significantly reduced in number, by 7 days after NMDA application. No differences in Brn3a and γ-synuclein labelling were detected in eyes pre-treated with creatine. Scale bar = 100 µm. *GCL*, ganglion cell layer; *INL*, inner nuclear layer; *ONL*, outer nuclear layer.

Effect of creatine on RGC loss due to high pressure retinal ischaemia

Establishment of retinal ischaemia via acute elevation of IOP also caused significant reduction in numbers of RGCs after 7 days. Electroretinogram traces recorded from rats at 7 days after IOP elevation show significant loss of b-waves whilst preserving a-wave, when compared to baseline (Figure 3.12). There is substantial loss of a-wave amplitude (40%) in response to IOP elevation. Treatment with creatine did not significantly preserve the b-wave amplitude (as percentage of baseline) of rat eyes after receiving IOP elevation (21.2% $\pm$ 1.5%, n = 8; vs. 20.6% $\pm$ 1.2%, n = 7; in control and creatine-treated eyes, respectively).

The effect of creatine on RGC survival in these animals is shown in Figure 3.13. No significant difference could be found between control and creatine-fed groups when comparing numbers of either Brn3a- or γ-synuclein-immunoreactive cells (Figure 3.13; $P > 0.05$). When the distribution of RGCs were stratified according to distance from the optic nerve head (central, middle and peripheral), no significant differences were observed for either antigen (Figure 3.14).

Histological sections of eyes treated with high pressure retinal ischaemia

Histological sections of retina treated with 75 minutes of high pressure ischaemia revealed significant loss of cells in the ganglion cell layer as well as marked thinning of the inner plexiform and nuclear layer, when assessed after 7 days (Figure 3.15).

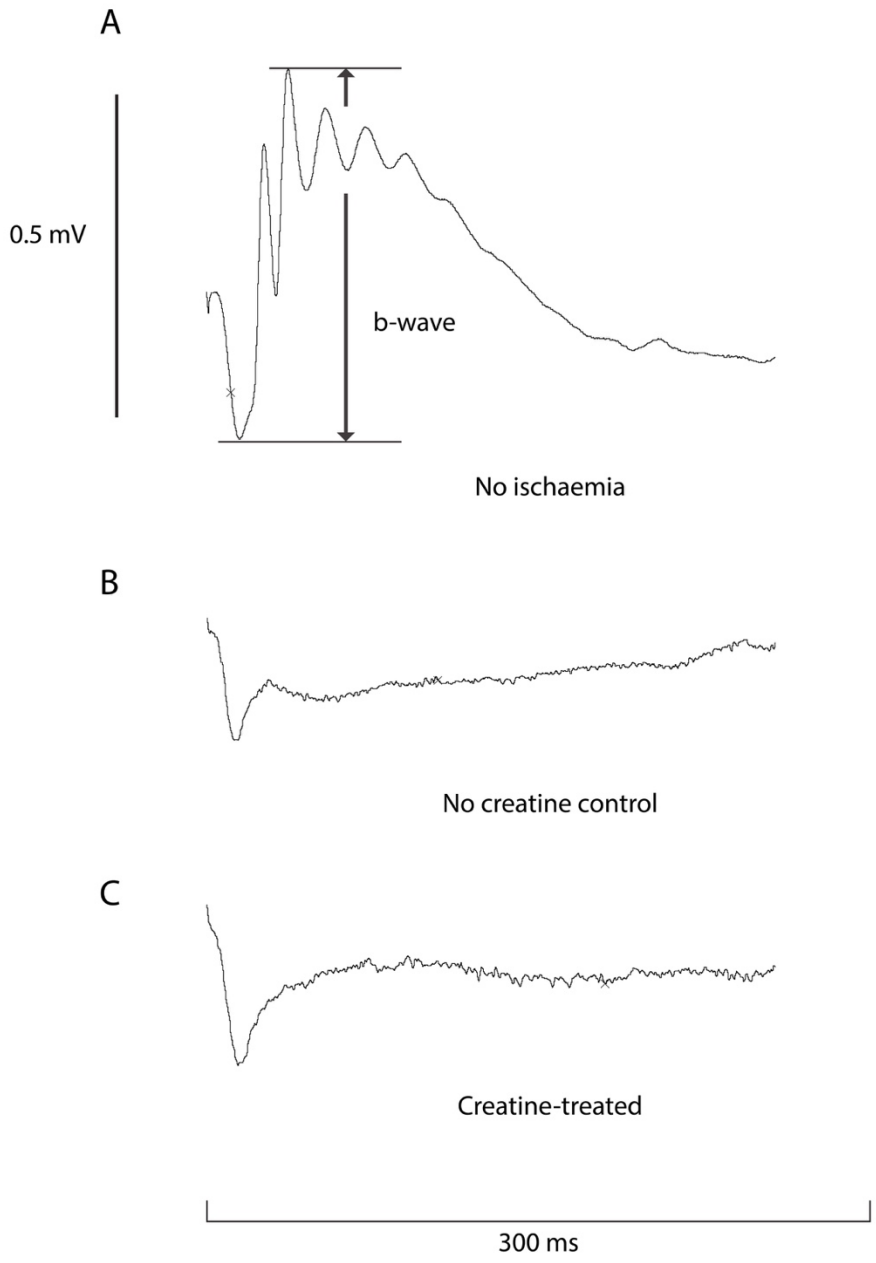

Figure 3.12. Representative electroretinograms at baseline (A) and at 7 days after 75 minutes of high intraocular pressure-induced retinal ischaemia in no-creatine control (B) and creatine-treated eyes (C), showing significant loss of b-wave and moderately preserved a-wave in both creatine-treated and control groups.

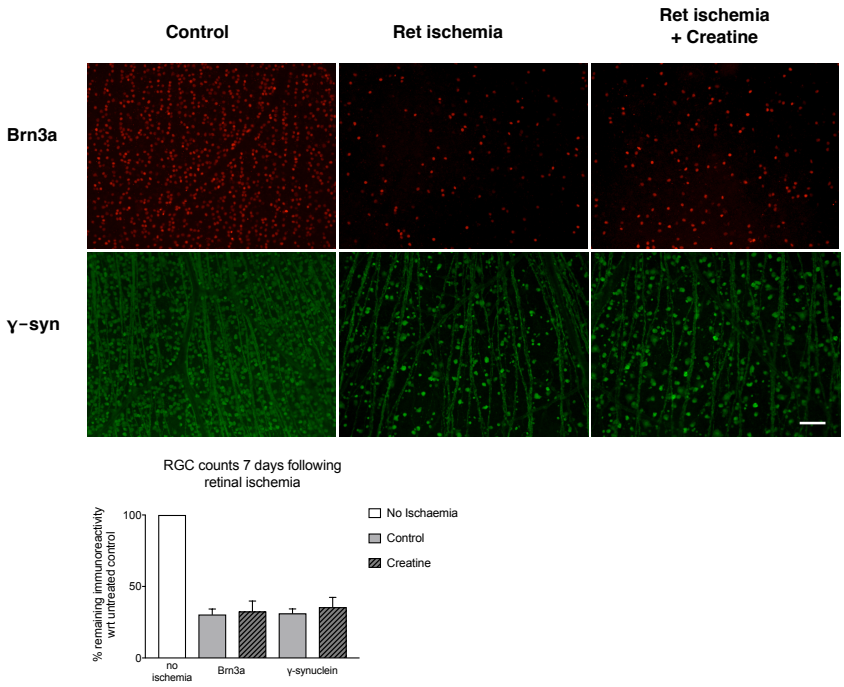

Figure 3.13. High intraocular pressure-induced retinal ischaemia (75 min) led to significant loss of both Brn3a-positive and γ-synuclein-positive cells. Prophylactic treatment with oral creatine did not result in any significant protection (Student T-test; n = 7-8). Scale bar = 100 µM.

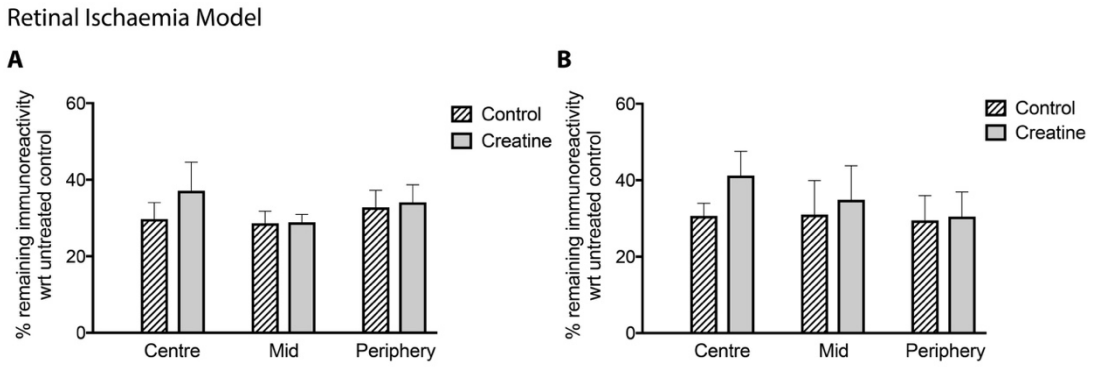

Figure 3.14. Summary of Brn3a- (A) and γ-synuclein-immunoreactive (B) cell loss, stratified based on retinal location into central, middle and peripheral counts, in both control and creatine-treated groups in NMDA-treated eyes. No statistical significant differences were found in any control-creatine comparison groups (Student T-test, n = 7-8).

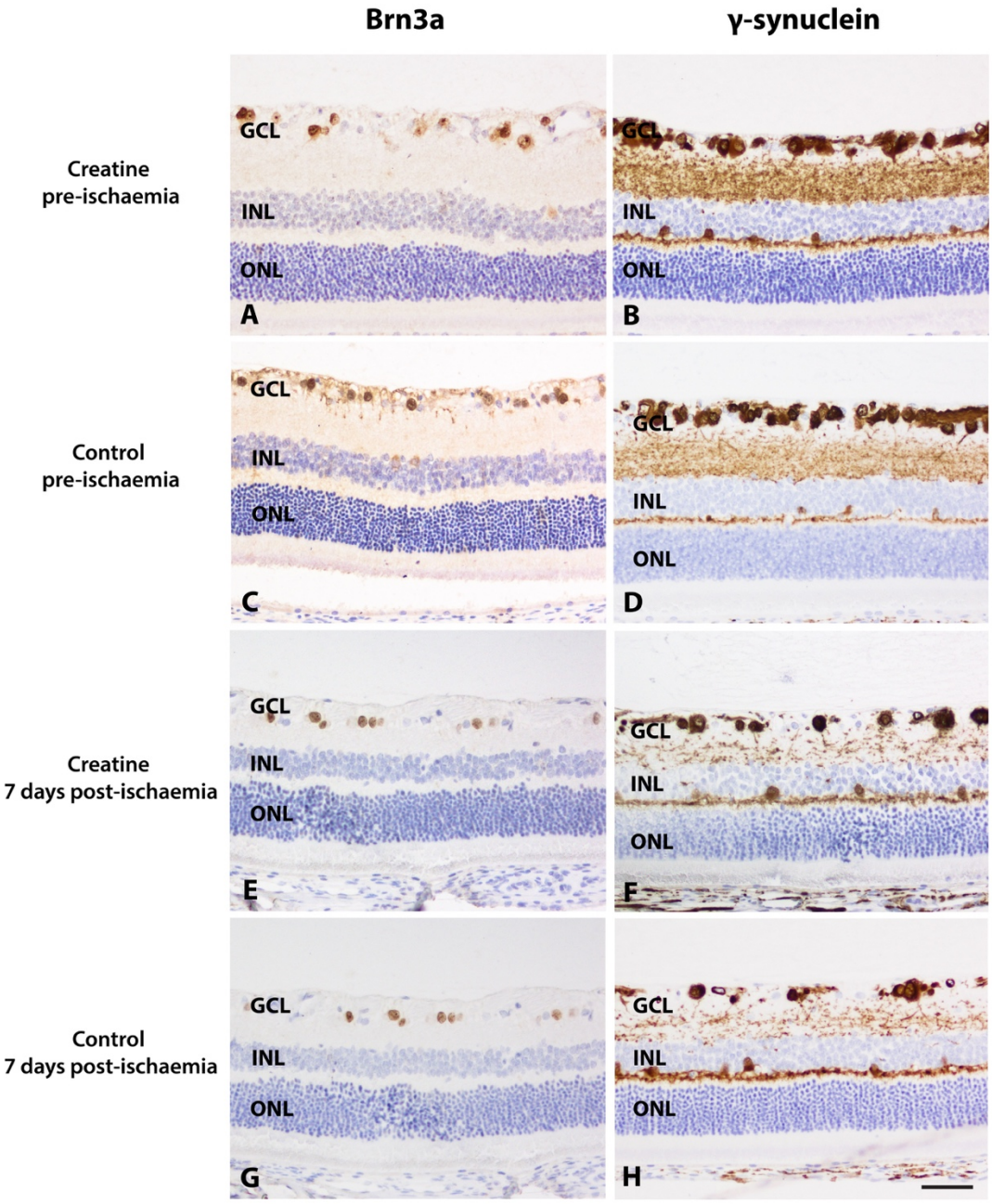

Figure 3.15. Photomicrographs showing retinal histology in tissue cross-sections labelled with Brn3a and γ-synuclein for both control/creatine eyes (A-D) and eyes fixed at 7 days following elevation of IOP (E-H). Elevation of IOP caused significant cell loss in the ganglion cell layer and thinning of the inner plexiform and nuclear layer. No differences in either Brn3a and γ-synuclein labelling were detected in eyes pre-treated with creatine. Scale bar = 100 µm. *GCL*, ganglion cell layer; *INL*, inner nuclear layer; *ONL*, outer nuclear layer.

3.4 Discussion

Cultures

In this study, we conducted several experiments to assess whether creatine was able to protect retinal neurons from insults that could affect cellular metabolism. Initial studies employed rat retinal cultures in which a metabolic dysfunction was induced by using sodium azide to compromise mitochondrial oxidative phosphorylation. We previously determined in rat retinal cultures that sodium azide could reliably induce neuronal loss via oxidative stress, mitochondrial membrane disruption and energetic dysfunction.[151] In the present study, sodium azide caused significant neuron loss in both acute and chronic models of metabolic dysfunction in cultured retinal cells. When creatine was added prophylactically to see if it would protect against sodium azide in the cultures, significant and concentration-dependent neuronal protection was observed when assessing numbers of both calretinin-positive and GABA-positive cells. Both calretinin and GABA have been used as reliable markers which label distinct neuron sets in rat retinal cultures: the former labels populations of amacrine cells and RGCs and the latter labels some amacrine cells only. Observed neuronal protection is consistent with other in vitro studies which demonstrate positive effects of creatine in paradigms of central nervous system neurodegenerative diseases.[76,91,96,153,154]

Animal Models

Having observed protection in vitro, we investigated whether creatine was able to protect retinal neurons in vivo. Two model systems were investigated: the application of NMDA to stimulate excitotoxic neurodegeneration and the implementation of retinal ischaemia by elevating IOP above systolic blood pressure. Both NMDA-induced excitotoxicity and high IOP-induced retinal ischaemia have been used widely as models of RGC injury which relate to glaucoma.[43,63,155-158]

Administration of exogenous NMDA in vivo has proven a reliable model in the study of neurodegenerative diseases since excitatory neurotoxicity has been proposed as one of the key mechanisms of injury in ocular neurodegenerative conditions, including glaucoma, retinal vessel occlusions and diabetic retinopathy.[65,159,160] It has been observed that within the CNS, accumulation of glutamate in the extracellular space leads to neuronal cell death via various pathways that may occur in parallel and result in both ongoing depolarization and self-propagating injury.[42,161,162] Stimulation of depolarization via excitatory neurotransmitters (namely glutamate) results in voltage-dependent Na^+ influx followed by passive influx of Cl^-. Water diffuses into neurons along the osmotic gradient and can cause osmotic shock and cell lysis – this type of cell death is categorised as *necrosis*. At the same time, intracellular Ca^{2+} levels rise due to multiple factors that both enhance Ca^{2+} influx and reduce Ca^{2+} efflux. Such factors include activation of receptors or channels that allow direct entry of Ca^{2+} into the cell, depletion of ATP due to the widespread over-action of receptors, mitochondrial dysfunction, and failure of Ca^{2+} extrusion secondary to ATP depletion. Increases in intracellular Ca^{2+} levels in turn cause activation of destructive enzymes such as calcium-activated neutral proteases (calpains), production of free radicals, mitochondrial injury and further release of glutamate from nerve terminals. These processes make up the delayed *apoptosis*, also characteristic of excitotoxic challenge, particularly when using NMDA versus other ionotropic glutamate receptor agonists such as kainate.[163,164] These described processes are in line with a previously proposed hypothesis that considered excessive intracellular Ca^{2+} as a trigger for neuronal death, namely "calcium loading" hypothesis.[65,165] NMDA receptors in the rat retina have been localized on RGCs and displaced amacrine cells in the ganglion cell layer, as well as in a population of amacrine cells located in the inner nuclear layer.[137]

Neuronal excitotoxicity also likely plays a major role in the pathogenesis of injury following retinal ischaemia.[63,112,166,167] Retinal ischaemia causes disturbances in neuronal metabolism via deprivation of energy, which in turn causes release of

neurotransmitters (especially glutamate) as ATP levels decline and membrane-based pumps can no longer function. These processes therefore produce a self-reinforcing cycle of neuronal excitation and energy depletion. Moreover, oxidative stress occurs in parallel as a result of ischaemia as mitochondrial function is compromised and excess free radicals are produced and remain un-detoxified. Furthermore, reperfusion injury can play a role in tissue damage; this occurs when an ischaemic tissue is re-oxygenated. This process is somewhat paradoxical in that the reperfusing oxygen would be expected to restore physiological functioning of the tissue but, in fact, radicals are principally formed when reduced compounds (accumulated during ischaemia) are re-oxidized. The free radical burst produced during early stage reperfusion subsequently overwhelms normal antioxidant mechanisms and leads to oxidative stress-related injury.[63]

In our study, we used Brn3a and γ-synuclein as markers for RGCs. Prophylactic treatment on rats did not result in any significant protection against either NMDA-stimulated excitotoxic injury or elevated IOP-induced retinal ischaemia. This was evident when total Brn3a-positive and γ-synuclein-positive cells were quantified and compared in treated versus non-treated retinas. Counts were compared either as a whole or for distinct retinal regions, as defined by relative distance from the optic nerve head (central, middle, peripheral); no significant difference was seen in creatine plus NMDA-treated retinas versus NMDA alone or in creatine plus ischaemia versus ischaemia alone.

With each series of experiments comparisons of levels of remaining labelled cells were undertaken after 7 days of treatment. In the case of the NMDA-treated animals, cell death is rapidly induced and we determined that 65% of RGCs were dead after 7 days, meaning that it was possible that even if creatine had slowed the rate of cell decline, its protective effect had been exhausted by this point. We thus also determined the effect of creatine on NMDA-induced RGC death by quantifying levels of apoptosis (by the appearance of TUNEL-labelling of cells in the RGC layer) at 8 hours, when this process is known to be at or near maximum.[124] When we analysed

NMDA-injured samples after 8 hours, 60% of RGCs were lost, which corresponded to significant TUNEL staining. At this time point, prophylactic treatment with creatine also did not show any significant differences in both Brn3a and TUNEL counts.

Malcon and colleagues treated Sprague-Dawley rats with 1% creatine for 1 week before and after subjecting them to intracerebral injections of NMDA.[94] When brain tissue was analysed one week after NMDA injection, significant reduction in volume of NMDA-induced striatal lesions (about 20%) was observed in the creatine-treated brains compared to controls (n = 11–12 each group). They also showed that the addition of nicotinamide provided additive protective effects against malonate-induced excitotoxic neuronal death.[94] On the other hand, in a recent study, intracerebral β-amyloid injections were performed on Wistar rats before feeding these animals on a diet containing 2% (w/w) creatine for a total of six weeks. At the end of the experiment, several tests including a measure for learning and memory retrieval, as well as TUNEL staining on histological sections, did not show any significant difference between the creatine-treated and control groups.[168]

Mechanisms of Protection

We showed in our in vitro studies that creatine was able to reduce both oxidative stress levels and incidences of apoptosis. The presented data were in agreement with previously proposed mechanisms for creatine in studies related to models of other neurodegenerative diseases.[71,75-82,91,94,96,153,154,169-172] We did not, however, detect that creatine was able to elevate cellular ATP levels in this study. Previous studies have not clearly detected higher ATP levels in cultured neurons treated with creatine, but did suggest a role of creatine in neuroprotection as an energy buffer. In 2007, Prass and colleagues, using magnetic resonance imaging, found an augmented cerebral blood flow and neuroprotection in creatine-fed mice in a rodent model of cerebral ischaemia.[171] Wilken and colleagues treated rat mothers with anoxia for 30 minutes and found that in creatine-fed animals subjected to the same insult, histological brain slices of their pups showed a significantly higher ATP levels.[92] On the other hand, Brewer and colleagues incubated hippocampal

neurons with creatine and showed protection against glutamate-induced excitotoxicity, but did not find a significantly higher ATP levels in the neuronal tissues.[96] However, their study did show that at the early stages of glutamate exposure, the phosphocreatine-to-ATP ratio was significantly higher in creatine-treated neurons, although the creatine levels, themselves, were lower. They proposed that this change in creatine or phosphocreatine levels was due to initial conversion of new intracellular creatine to phosphocreatine with the consumption of ATP (and rise in phosphocreatine). It was thought that these early changes in creatine and phosphocreatine levels turned creatine into an energy buffer instead of an energy supply. Furthermore, energy consumption may easily exceed the ability of the cell to regenerate high-energy phosphates, hence there was no difference in ATP levels in both their study and our study.[96]

Explanation for non-protection In Vivo

It is well established that mitochondrial compromise via treatment with sodium azide leads to elevated cellular oxidative stress levels and increased apoptosis.[151] We have shown that creatine significantly reduces apoptosis as well as oxidative stress in culture. It is interesting that the culture model in this study showed a protection from creatine, but the protection was not evident in either of the two animal models. One explanation is that the two types of RGC injury were too drastic such that we were unable to detect any putative protection afforded by creatine. Both high IOP-induced retinal ischaemia and NMDA-induced retinal excitotoxicity are extremely acute models of retinal damage, instead of slow-progressing and chronic low-grade damage, such as a disease such as glaucoma. If creatine does provide a small amount of protection, perhaps it would be more evident in a model where damage is less pronounced and of a more protracted duration.

Another explanation for the lack of effect seen by analysing RGC quantification in the animal models is that the prophylactic treatment of creatine, as applied via the oral route, may not provide a sufficient end-point concentration of this compound in the retina (i.e. insufficient dose). Compared to creatine

neuroprotection studies in other neurodegenerative disease models, however, it would be assumed that the concentration used in our study was sufficient (2% w/w in chow). In a Parkinson model, for example, pretreatment of mice with 1% creatine (w/w in chow) for 2 weeks prior to MPTP injections almost completely abolished deleterious effects to dopaminergic neurons.[77] Similarly, in studies in a rodent model of ALS, a dose-dependent increase in survival compared to placebo (reduction in neuronal cell death) was observed with 1-2% of creatine (in chow), when started within 1-2 months post-partum.[79]

Future Directions

The potential use of creatine as a neuroprotective compound for the retina requires more exploration with further studies, both in vitro and in vivo. Culture models that implement different types of insult, e.g. oxidative insults such as with the use of hydrogen peroxide, can be attempted. Instead of a mixed retinal culture, homogeneous cultures of RGCs alone can be utilized to examine the effect of creatine on the morphology of the cell with or without metabolic insults. However, to date, isolated RGC cultures are unable to consistently produce enough cells for quantification purposes in neuroprotection studies. Further mechanistic studies can be performed to allow greater characterisation of the effects of creatine in retinal cultures, such as tests for ATP levels and mitochondrial membrane potentials. In animal studies, different models of RGC damage can be employed: for instance, experimental glaucoma models that more readily mimic the disease by providing a longer duration of low-grade high IOP-induced RGC damage.

3.5 Conclusion

In conclusion, prophylactic creatine treatment provided neuroprotection in a culture model of energetic dysfunction in the retina, but was not protective for retinal ganglion cells in animal models of excitotoxicity. This discrepancy in its ability to provide neuroprotection needs to be further explored and validated. Nonetheless, mechanistic studies have shown that creatine displays antioxidant and anti-apoptotic qualities. Further mechanistic studies need to be performed to fully characterize effects of creatine in the retina.

4 Summary and Discussion

4.1 Introduction

Neurodegeneration of the retina accounts for a large group of retinal pathologies, including glaucoma, age-related macular degeneration and diabetic retinopathy.[4,53,173,174] These diseases are largely managed by controlling known risk factors, such as serum glucose levels for diabetic patients and intraocular pressure for glaucoma patients. Despite having in-depth understanding (or theories) of the process of neuronal degeneration at the molecular level, there exist little means to directly deal with these processes, such as oxidative stress and mitochondrial membrane potential instability. In the clinical setting, there are a significant proportion of cases where dealing with known risk factors still results in neurodegeneration, as is seen in the case of glaucoma.[39,40] There are several potential agents for potential application of neuroprotection that have been suggested in the literature, including the guanidine compound creatine, which has recently been studied and shown to be neuroprotective in models of major neurodegenerative diseases of the central nervous system.[42,73,91] This study explored the prospect of using creatine as a neuroprotective agent in the setting of neurodegeneration in the retina. We first performed a study to compare different markers of retinal ganglion cells in a well-known NMDA-induced excitotoxicity model. Then, we looked at the effect of creatine in cultures in a model of metabolic dysfunction. Lastly, we investigated the effect of creatine on retinal ganglion cells in animal models of excitotoxicity and retinal ischaemia.

4.2 Summary of experimental findings

In the first study, we described that a reliable and reproducible marker for determining loss of RGCs in relevant retinal toxicity models was required, especially when the effect of potential protective compounds was to be analysed, such as was

to be the case for creatine in our work. We therefore employed immunohistochemical labelling of retinal whole-mounts and tested a variety of antibody markers known to label RGCs for reproducibility, specificity, accurate dissemination of cell loss and good signal-to-noise ratio. Data showed that intravitreal injection of NMDA to induce retinal toxicity leads death of at least 90% of the RGC population within the first 24 hours when quantifying cells labelled by Brn3a, RBPMS and γ-synuclein in the ganglion cell layer. Losses of Brn3a- and RBPMS-positive cells were the most marked, and these labels were the most specific, had the best signal-to-noise ratios and had the best agreement with previously published cell loss dynamics in this model; furthermore, their proportion of cell loss was similar to each other. γ-synuclein- and calretinin-positive cells, in comparison, showed approximately 3 times more surviving neurons by 24 hours as well as 7 days post-NMDA exposure. From our study, it was clear that there was less variability between counts across different time points up to 7 days post NMDA for Brn3a, which makes it perhaps a more reliable marker for studies that require quantification analyses on different time-points post-RGC injury. We therefore selected this marker for studies analyzing the potential for creatine to provide neuroprotection to RGCs, in our following in vivo studies. In our second study, we looked at the effect of creatine on retinal neurons in several models of neuronal insult. In a culture model of metabolic dysfunction, we showed that sodium azide was able to cause a loss of up to 60-86% of neurons and also that prophylactic treatment with creatine resulted in significant protection of up to 50% of this loss. Assessment of the level of reactive oxygen species in this model e showed that sodium azide produces oxidative stress that was significantly reduced by prophylactic treatment with creatine. The final ATP content however was no different when comparing creatine and non-creatine treated cultures, further supporting the role of creatine role as an energy buffer, not as the supplier of an additional energy substrate. When the effects of creatine were tested in animal models of RGC injury. However, by assessing Brn3a-immunoreactive cell counts in labelled retinal whole-mounts, no protection was found. This was the case after both intravitreal injections of NMDA when analysed at either 8 hours or 7 days,

or in a model of retinal ischaemia induced by acute elevation of IOP. The mismatch between the in vitro and in vivo findings for the protective effect of creatine were speculated upon.

4.3 Overall significance

We have shown that NMDA administration results in retinal neuronal damage maximally within the first 24 hours and the rate of damage reduces from then on. There are no gold standard cell markers for RGCs for quantification purposes, but Brn3a may be a more sensitive marker for quantification of cells at and beyond 24 hours after retinal injuries that target RGCs.

The current body of work provides further evidence that sodium azide creates a reproducible metabolic insult to retinal neurons and that one of its mechanisms is via the induction of oxidative stress. Oxidative stress is proposed to be a major mechanistic component contributing to neurodegeneration in general and sodium azide can therefore be considered as a reliable test agent to provide such an insult, in furtur neurodegeneration or neuroprotection studies, especially when used in in vitro preparations.

Of novel insight to the literature, we showed that creatine was able to lower azide-induced oxidative stress and was able to protect retinal neurons from damage resulting from this compound. This further strengthens the claims of creatine being able to act as a neuroprotective agent in the central nervous system. The study also supports previous notions regarding the mechanism of action for the protection of neurons by creatine, including stabilization of the mitochondrial membrane potential and acting as a spatial and temporal energy shuttle. By stabilizing the mitochondrial permeability pore and buffering energy within the cell during periods of stress, the resultant effect would involve reduction in cellular free radical damage.[73,91]

Clinically, creatine is a compound that can be easily administered – mainly orally – and bears very minimal side-effects. Any protection that is found in in vitro and in vivo studies can readily be explored in human trials. The difficulty, however, may lie in the timing of when creatine supplementation should start and the mode and dose of administration. It was suggested that neuroprotection with creatine hinges on the prophylactic nature of its systemic supplementation, and this may be difficult to apply in retinal diseases that features a period of subclinical pathological neurodegeneration. Even if creatine can be started early on, the next question would then be how early should creatine be started?

4.4 Limitations

Despite showing protection in a culture model of metabolic dysfunction, creatine did not show any protection in two distinct animal models of RGC injury. This may be for several reasons. Firstly, retinal ischaemia and intravitreal administration of NMDA both provide quick and strong insults to retinal neurons. It may be possible that the speed and magnitude of neuronal stress causes a tissue damage profile that is beyond being able to be protected by creatine, or indeed, any other compound. Secondly, we did not test the effect of creatine on RGC survival at different doses – especially lower doses – of NMDA, or at a shorter duration of high IOP-induced retinal ischaemia. The in vivo models tested in our study killed as many as 70% of the RGCs, which may be too high for creatine to protect, especially if its potential neuroprotective efficacy was of low magnitude. Thirdly, it may be possible that the amount of creatine administered in vivo may not be enough to reach a significant level for neuroprotection. Although in previous studies of major neurodegenerative diseases, creatine at concentrations of 1-2% (w/w in chow) was used, as in our study, and this had achieved neuroprotection, this may not be enough for protection within the retina.[94] Fourthly, prophylactic creatine treatment in this work may not have been applied early enough before induction of retinal injury to reach a suitable and

effective treatment level. However, this may be unlikely given that in other neurodegeneration studies that had shown protection, creatine was administered as late as 1-2 weeks prior to neuronal insults.[79,80,94] On the other hand, serum creatine has been shown to have a short half-life of 3-5 hours, which supposes the need for a more frequent administration of creatine at a higher amount to provide sufficiently high levels of creatine in the system to show neuroprotection.[175] Analysis of tissue creatine levels may inform us whether oral creatine administration is successful at increasing creatine levels in the retina.

One limitation for our study was the lack of positive controls to compare creatine to. However, it is difficult to search for a neuroprotectant that has the same mechanism of action as creatine in protecting the retina from various insults. For example, MK801 has been shown to provide neuroprotection in the retina, but with a different mechanism of protection to creatine, in that it functions as a non-competitive NMDA-receptor blocker.[176] A positive control was therefore not chosen because it will be unlikely for another neuroprotective agent to be a true positive control to creatine.

Another limitation for our study was the method of creatine administration. We used oral feeds for creatine to be administered because it was proven to be effective in other studies and also because oral feeds are easy to implement and easier to translate to human trials. Other options for creatine administration include intravitreal and subconjunctival injection of creatine, which provides direct contact of creatine with the retinal neurons (especially the ganglion cell layer). However, such methods would be difficult to achieve clinical relevance due to the invasiveness of intravitreal or subconjunctival injections.

4.5 Future directions

This body of work sets a stage for further exploration on the mechanisms of action of creatine and its potential neuroprotective qualities. Certainly, aspects of energy buffering, mitochondrial stabilization and anti-apoptotic qualities can be more extensively investigated in the context of retinal neurodegeneration. Models of other types of metabolic dysfunction can be implemented to fully elucidate creatine's effects on retinal neurons. Certainly, cultures of specific, rather than mixed populations of cells, such as RGCs, could be employed to look at effects of creatine on specific cell types. However, single cell type cultures of the retina are often problematic to establish and cell yield is often not enough for quantification purposes. Aspects of fine-tuning of the creatine concentration to be administered as well as the timing (prophylactic duration) can also be investigated in both culture and animal models. For in vivo work, there are a large variety of types of injury models as well as types of retinal neurons to focus on, but perhaps a better step to take is to use reliable models of oxidative stress and energy failure at a much lower magnitude of injury. Rats and mice remain useful animals for experimentation as they are inexpensive and easy to house. Although there is a high degree of conservation between rodent and human genomes, the anatomy of the rodent retina is not similar to humans in that there is an absence of lamina cribrosa in the optic nerve and the very small globe size makes it hard to access clinically.[177] Monkeys have close phylogeny and high homology with humans, having retinal and optic nerve anatomy that is almost identical, also making them an excellent model for retinal experimentation. Unfortunately, they are very expensive, their availability is limited, and they are difficult to handle.[178,179]

Another potential future investigative strategy is to assess the effect of creatine in addition to other proposed neuroprotective agents in protecting retinal neurons. We have previously shown that hyperglycaemia results in neuroprotection of retinal ganglion cells from an ischemic insult.[68,69] Although mechanisms of protection were not yet explored, glucose was proposed to contribute to cellular bioenergetics in the

context of retinal mitochondrial and energetic failure. It is also possible that glucose may work in a separate pathway to reduce oxidative stress. Coenzyme Q10 is a component of the mitochondrial respiratory pathway that carries electrons from complexes I and II to complex III, and is also a potent anti-oxidant. Supplementation of CoQ10 has been shown to protect in Huntington's disease and Parkinson's disease. In vitro research has indicated that oxidative stress-induced apoptosis is alleviated by CoQ10 supplementation through the stabilization of the mitochondrial membrane potential. The neuroprotective effect of CoQ10 has also been demonstrated in a high intraocular pressure glaucoma model.[42,75,106,150,180,181]

4.6 Conclusions

In conclusion, results from this body of work allow scope for more research on whether creatine is able to provide neuroprotection in retinal neurodegenerative diseases. Creatine is able to achieve partial neuroprotection in the retina from metabolic dysfunction – likely through reduction in oxidative stress and prevention of cellular apoptosis. This protection, however, is yet to be seen in animal studies.

5 References

1. Baden T, Berens P, Franke K, Rosón MR, Bethge M, Euler T. The functional diversity of retinal ganglion cells in the mouse. *Nature* 2016; **529**: 345-50.
2. Sanes JR, Masland RH. The types of retinal ganglion cells: current status and implications for neuronal classification. *Annu Rev Neurosci* 2015; **38**: 221-46.
3. Masland RH. The neuronal organization of the retina. *Neuron* 2012; **76**: 266-80.
4. Schmidt K-G, Bergert H, Funk R. Neurodegenerative diseases of the retina and potential for protection and recovery. *Curr Neuropharmacol* 2008; **6**: 164-78.
5. Sun W, Li N, He S. Large-scale morphological survey of mouse retinal ganglion cells. *J Comp Neurol* 2002; **451**: 115-26.
6. Eggers ED, Lukasiewicz PD. Multiple pathways of inhibition shape bipolar cell responses in the retina. *Vis Neurosci* 2011; **28**: 95-108.
7. Taylor W, Smith R. Trigger features and excitation in the retina. *Curr Opin Neurobiol* 2011; **21**: 672-78.
8. Sun W, Deng Q, Levick WR, He S. ON direction-selective ganglion cells in the mouse retina. *J Physiol* 2006; **576**: 197-202.
9. Coombs J, Van Der List D, Wang G-Y, Chalupa L. Morphological properties of mouse retinal ganglion cells. *Neuroscience* 2006; **140**: 123-36.
10. Dacey DM, Peterson BB, Robinson FR, Gamlin PD. Fireworks in the primate retina: in vitro photodynamics reveals diverse LGN-projecting ganglion cell types. *Neuron* 2003; **37**: 15-27.
11. Wang L, Dong J, Cull G, Fortune B, Cioffi GA. Varicosities of intraretinal ganglion cell axons in human and nonhuman primates. *Invest Ophthalmol Vis Sci* 2003; **44**: 2-9.
12. Bristow EA, Griffiths PG, Andrews RM, Johnson MA, Turnbull DM. The distribution of mitochondrial activity in relation to optic nerve structure. *Arch Ophthal* 2002; **120**: 791-96.

13. Funk R. Blood supply of the retina. *Ophthalmic Res* 1997; **29**: 320-25.
14. Moreno MC, Campanelli J, Sande P, Sáenz DA, Sarmiento MIK, Rosenstein RE. Retinal oxidative stress induced by high intraocular pressure. *Free Radic Biol Med* 2004; **37**: 803-12.
15. Osborne N, Lascaratos G, Bron A, Chidlow G, Wood J. A hypothesis to suggest that light is a risk factor in glaucoma and the mitochondrial optic neuropathies. *Br J Ophthalmol* 2006; **90**: 237-41.
16. Kortuem K, Geiger LK, Levin LA. Differential susceptibility of retinal ganglion cells to reactive oxygen species. *Invest Ophthalmol Vis Sci* 2000; **41**: 3176-82.
17. Fortune B, Bui BV, Morrison JC, Johnson EC, Dong J, Cepurna WO et al. Selective ganglion cell functional loss in rats with experimental glaucoma. *Invest Ophthalmol Vis Sci* 2004; **45**: 1854-62.
18. Osborne N, Chidlow G, Layton C, Wood J, Casson R, Melena J. Optic nerve and neuroprotection strategies. *Eye* 2004; **18**: 1075-84.
19. Osborne NN, Chidlow G, Wood JP, Schmidt K-G, Casson R, Melena J. Expectations in the treatment of retinal diseases: neuroprotection. *Curr Eye Res* 2001; **22**: 321-32.
20. Barnstable C, Dräger U. Thy-1 antigen: a ganglion cell specific marker in rodent retina. *Neuroscience* 1984; **11**: 847-55.
21. Schmued LC, Stowers CC, Scallet AC, Xu L. Fluoro-Jade C results in ultra high resolution and contrast labeling of degenerating neurons. *Brain Res* 2005; **1035**: 24-31.
22. Chidlow G, Wood JP, Sarvestani G, Manavis J, Casson RJ. Evaluation of Fluoro-Jade C as a marker of degenerating neurons in the rat retina and optic nerve. *Exp Eye Res* 2009; **88**: 426-37.
23. Kwong JM, Caprioli J, Piri N. RNA binding protein with multiple splicing: a new marker for retinal ganglion cells. *Invest Ophthalmol Vis Sci* 2010; **51**: 1052-58.

24. Badea TC, Cahill H, Ecker J, Hattar S, Nathans J. Distinct roles of transcription factors brn3a and brn3b in controlling the development, morphology, and function of retinal ganglion cells. *Neuron* 2009; **61**: 852-64.
25. Surgucheva I, Weisman AD, Goldberg JL, Shnyra A, Surguchov A. γ-Synuclein as a marker of retinal ganglion cells. *Mol Vis* 2008.
26. Xiang M, Zhou L, Macke J, Yoshioka T, Hendry SH, Eddy R et al. The Brn-3 family of POU-domain factors: primary structure, binding specificity, and expression in subsets of retinal ganglion cells and somatosensory neurons. *J Neurosci* 1995; **15**: 4762-85.
27. Rogers J, Khan M, Ellis J. Calretinin and other CaBPs in the nervous system. *Advances in experimental medicine and biology* 1990; **269**: 195-203.
28. Nadal-Nicolás FM, Jiménez-López M, Sobrado-Calvo P, Nieto-López L, Cánovas-Martínez I, Salinas-Navarro M et al. Brn3a as a marker of retinal ganglion cells: qualitative and quantitative time course studies in naive and optic nerve–injured retinas. *Invest Ophthalmol Vis Sci* 2009; **50**: 3860-68.
29. Rodriguez AR, Müller S, Pérez L, Brecha NC. The RNA binding protein RBPMS is a selective marker of ganglion cells in the mammalian retina. *J Comp Neurol* 2014; **522**: 1411-43.
30. Casson RJ, Chidlow G, Ebneter A, Wood JP, Crowston J, Goldberg I. Translational neuroprotection research in glaucoma: a review of definitions and principles. *Clin Exp Ophthalmol* 2012; **40**: 350-57.
31. Peters D, Bengtsson B, Heijl A. Lifetime risk of blindness in open-angle glaucoma. *Am J Ophthalmol* 2013; **156**: 724-30.
32. Quigley HA, Broman AT. The number of people with glaucoma worldwide in 2010 and 2020. *Br J Ophthalmol* 2006; **90**: 262-67.
33. Tielsch JM, Sommer A, Katz J, Royall RM, Quigley HA, Javitt J. Racial variations in the prevalence of primary open-angle glaucoma: the Baltimore Eye Survey. *Jama* 1991; **266**: 369-74.
34. Sommer A, Tielsch JM, Katz J, Quigley HA, Gottsch JD, Javitt J et al. Relationship between intraocular pressure and primary open angle

glaucoma among white and black Americans: the Baltimore Eye Survey. *Arch Ophthal* 1991; **109**: 1090-95.

35. Harwerth RS, Carter-Dawson L, Shen F, Smith EL, Crawford M. Ganglion cell losses underlying visual field defects from experimental glaucoma. *Invest Ophthalmol Vis Sci* 1999; **40**: 2242-50.
36. Sommer A, Katz J, Quigley HA, Miller NR, Robin AL, Richter RC et al. Clinically detectable nerve fiber atrophy precedes the onset of glaucomatous field loss. *Arch Ophthal* 1991; **109**: 77-83.
37. Harwerth R, Quigley H. Visual Field Defects and Retinal Ganglion Cell Losses in Human Glaucoma Patients. *Invest Ophthalmol Vis Sci* 2004; **45**: 3473-73.
38. Yanagi M, Kawasaki R, Wang JJ, Wong TY, Crowston J, Kiuchi Y. Vascular risk factors in glaucoma: a review. *Clin Exp Ophthalmol* 2011; **39**: 252-58.
39. Lee S, Van Bergen NJ, Kong GY, Chrysostomou V, Waugh HS, O'Neill EC et al. Mitochondrial dysfunction in glaucoma and emerging bioenergetic therapies. *Exp Eye Res* 2011; **93**: 204-12.
40. Chrysostomou V, Rezania F, Trounce IA, Crowston JG. Oxidative stress and mitochondrial dysfunction in glaucoma. *Curr Opin Pharmacol* 2013; **13**: 12-15.
41. Kong GY, Van Bergen NJ, Trounce IA, Crowston JG. Mitochondrial dysfunction and glaucoma. *J Glaucoma* 2009; **18**: 93-100.
42. Schober MS, Chidlow G, Wood JP, Casson RJ. Bioenergetic‐based neuroprotection and glaucoma. *Clin Exp Ophthalmol* 2008; **36**: 377-85.
43. Osborne NN. Pathogenesis of ganglion "cell death" in glaucoma and neuroprotection: focus on ganglion cell axonal mitochondria. *Prog Brain Res* 2008; **173**: 339-52.
44. Fechtner RD, Weinreb RN. Mechanisms of optic nerve damage in primary open angle glaucoma. *Surv Ophthalmol* 1994; **39**: 23-42.
45. Ernest J. Pathogenesis of glaucomatous optic nerve disease. *Trans Am Ophthalmol Soc* 1975; **73**: 366.

46. Sigal IA, Ethier CR. Biomechanics of the optic nerve head. *Exp Eye Res* 2009; **88**: 799-807.
47. Sigal IA, Flanagan JG, Ethier CR. Factors influencing optic nerve head biomechanics. *Invest Ophthalmol Vis Sci* 2005; **46**: 4189-99.
48. Burgoyne CF, Downs JC, Bellezza AJ, Suh J-KF, Hart RT. The optic nerve head as a biomechanical structure: a new paradigm for understanding the role of IOP-related stress and strain in the pathophysiology of glaucomatous optic nerve head damage. *Prog Retin Eye Res* 2005; **24**: 39-73.
49. Burgoyne CF, Morrison JC. The anatomy and pathophysiology of the optic nerve head in glaucoma. *J Glaucoma* 2001; **10**: S16-S18.
50. Tezel G, Hernandez MR, Wax MB. In vitro evaluation of reactive astrocyte migration, a component of tissue remodeling in glaucomatous optic nerve head. *Glia* 2001; **34**: 178-89.
51. Nakazawa T, Nakazawa C, Matsubara A, Noda K, Hisatomi T, She H et al. Tumor necrosis factor-α mediates oligodendrocyte death and delayed retinal ganglion cell loss in a mouse model of glaucoma. *J Neurosci* 2006; **26**: 12633-41.
52. Yan X, Tezel G, Wax MB, Edward DP. Matrix metalloproteinases and tumor necrosis factor α in glaucomatous optic nerve head. *Arch Ophthal* 2000; **118**: 666-73.
53. Tezel G. Oxidative stress in glaucomatous neurodegeneration: mechanisms and consequences. *Prog Retin Eye Res* 2006; **25**: 490-513.
54. Sultan MB, Mansberger SL, Lee PP. Understanding the importance of IOP variables in glaucoma: a systematic review. *Surv Ophthalmol* 2009; **54**: 643-62.
55. Kaufmann C, Bachmann LM, Robert YC, Thiel MA. Ocular pulse amplitude in healthy subjects as measured by dynamic contour tonometry. *Arch Ophthal* 2006; **124**: 1104-08.

56. Flammer J, Orgül S, Costa VP, Orzalesi N, Krieglstein GK, Serra LM et al. The impact of ocular blood flow in glaucoma. *Prog Retin Eye Res* 2002; **21**: 359-93.

57. Kerr J, Nelson P, O'Brien C. A comparison of ocular blood flow in untreated primary open-angle glaucoma and ocular hypertension. *Am J Ophthalmol* 1998; **126**: 42-51.

58. Flammer J, Pache M, Resink T. Vasospasm, its role in the pathogenesis of diseases with particular reference to the eye. *Prog Retin Eye Res* 2001; **20**: 319-49.

59. Pournaras CJ, Rungger-Brändle E, Riva CE, Hardarson SH, Stefansson E. Regulation of retinal blood flow in health and disease. *Prog Retin Eye Res* 2008; **27**: 284-330.

60. Hayreh SS. Blood flow in the optic nerve head and factors that may influence it. *Prog Retin Eye Res* 2001; **20**: 595-624.

61. Hofman P, Hoyng P, Vrensen GF, Schlingemann RO. Lack of blood–brain barrier properties in microvessels of the prelaminar optic nerve head. *Invest Ophthalmol Vis Sci* 2001; **42**: 895-901.

62. Riva CE, Hero M, Titze P, Petrig B. Autoregulation of human optic nerve head blood flow in response to acute changes in ocular perfusion pressure. *Graefes Arch Clin Exp Ophthalmol* 1997; **235**: 618-26.

63. Osborne NN, Casson RJ, Wood JP, Chidlow G, Graham M, Melena J. Retinal ischemia: mechanisms of damage and potential therapeutic strategies. *Prog Retin Eye Res* 2004; **23**: 91-147.

64. Flammer J, Orgül S. Optic nerve blood-flow abnormalities in glaucoma. *Prog Retin Eye Res* 1998; **17**: 267-89.

65. Casson RJ. Possible role of excitotoxicity in the pathogenesis of glaucoma. *Clin Exp Ophthalmol* 2006; **34**: 54-63.

66. Osborne NN, del Olmo-Aguado S. Maintenance of retinal ganglion cell mitochondrial functions as a neuroprotective strategy in glaucoma. *Curr Opin Pharmacol* 2013; **13**: 16-22.

67. Brubaker RF. Targeting outflow facility in glaucoma management. *Surv Ophthalmol* 2003; **48**: S17-S20.

68. Shibeeb OS, Chidlow G, Han G, Wood JP, Casson RJ. Effect of subconjunctival glucose on retinal ganglion cell survival in experimental retinal ischaemia and contrast sensitivity in human glaucoma. *Clin Exp Ophthalmol* 2015.

69. Ebneter A, Chidlow G, Wood JP, Casson RJ. Protection of retinal ganglion cells and the optic nerve during short-term hyperglycemia in experimental glaucoma. *Arch Ophthal* 2011; **129**: 1337-44.

70. Moxon-Lester L, Takamoto K, Colditz PB, Barnett NL. S-adenosyl-L-methionine restores photoreceptor function following acute retinal ischemia. *Vis Neurosci* 2009; **26**: 429-41.

71. Brustovetsky N, Brustovetsky T, Dubinsky JM. On the mechanisms of neuroprotection by creatine and phosphocreatine. *J Neurochem* 2001; **76**: 425-34.

72. Nakashima T, Tomi M, Katayama K, Tachikawa M, Watanabe M, Terasaki T et al. Blood-to-retina transport of creatine via creatine transporter (CRT) at the rat inner blood–retinal barrier. *J Neurochem* 2004; **89**: 1454-61.

73. Wallimann T, Tokarska-Schlattner M, Schlattner U. The creatine kinase system and pleiotropic effects of creatine. *Amino acids* 2011; **40**: 1271-96.

74. Klopstock T, Elstner M, Bender A. Creatine in mouse models of neurodegeneration and aging. *Amino Acids* 2011; **40**: 1297-303.

75. Yang L, Calingasan NY, Wille EJ, Cormier K, Smith K, Ferrante RJ et al. Combination therapy with coenzyme Q10 and creatine produces additive neuroprotective effects in models of Parkinson's and Huntington's diseases. *J Neurochem* 2009; **109**: 1427-39.

76. Andres RH, Ducray AD, Pérez-Bouza A, Schlattner U, Krebs SH, Seiler RW et al. Creatine supplementation improves dopaminergic cell survival and protects against MPP+ toxicity in an organotypic tissue culture system. *Cell Transplant* 2005; **14**: 537-50.

77. Matthews RT, Ferrante RJ, Klivenyi P, Yang L, Klein AM, Mueller G et al. Creatine and cyclocreatine attenuate MPTP neurotoxicity. *Exp Neurol* 1999; **157**: 142-49.

78. Klivenyi P, Gardian G, Calingasan NY, Yang L, Beal MF. Additive neuroprotective effects of creatine and a cyclooxygenase 2 inhibitor against dopamine depletion in the 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease. *J Mol Neurosci* 2003; **21**: 191-98.

79. Andreassen OA, Jenkins BG, Dedeoglu A, Ferrante KL, Bogdanov MB, Kaddurah‐Daouk R et al. Increases in cortical glutamate concentrations in transgenic amyotrophic lateral sclerosis mice are attenuated by creatine supplementation. *J Neurochem* 2001; **77**: 383-90.

80. Klivenyi P, Ferrante RJ, Matthews RT, Bogdanov MB, Klein AM, Andreassen OA et al. Neuroprotective effects of creatine in a transgenic animal model of amyotrophic lateral sclerosis. *Nat Med* 1999; **5**: 347-50.

81. Ferrante RJ, Andreassen OA, Jenkins BG, Dedeoglu A, Kuemmerle S, Kubilus JK et al. Neuroprotective effects of creatine in a transgenic mouse model of Huntington's disease. *J Neurosci* 2000; **20**: 4389-97.

82. Matthews RT, Yang L, Jenkins BG, Ferrante RJ, Rosen BR, Kaddurah-Daouk R et al. Neuroprotective effects of creatine and cyclocreatine in animal models of Huntington's disease. *J Neurosci* 1998; **18**: 156-63.

83. Wallimann T, Tokarska‐Schlattner M, Neumann D, Epand RM, Epand RF, Andres RH et al. The phosphocreatine circuit: molecular and cellular physiology of creatine kinases, sensitivity to free radicals, and enhancement by creatine supplementation. *Mol Sys Bioenerg* 2007: 195-264.

84. McLeish MJ, Kenyon GL. Relating structure to mechanism in creatine kinase. *Crit Rev Biochem Mol Biol* 2005; **40**: 1-20.

85. O'Gorman E, Beutner G, Dolder M, Koretsky AP, Brdiczka D, Wallimann T. The role of creatine kinase in inhibition of mitochondrial permeability transition. *FEBS letters* 1997; **414**: 253-57.

86. Wallimann T, Hemmer W. Creatine kinase in non-muscle tissues and cells. In.*Creatine kinase in non-muscle tissues and cells*: Springer, 1994; 193-220.
87. Hemmer W, Wallimann T. Functional aspects of creatine kinase in brain. *Dev Neurosci* 1994; **15**: 249-60.
88. Wallimann T, Wyss M, Brdiczka D, Nicolay K, Eppenberger H. Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the'phosphocreatine circuit'for cellular energy homeostasis. *Biochemical Journal* 1992; **281**: 21.
89. Balestrino M, Lensman M, Parodi M, Perasso L, Rebaudo R, Melani R et al. Role of creatine and phosphocreatine in neuronal protection from anoxic and ischemic damage. *Amino Acids* 2002; **23**: 221-29.
90. Andres RH, Ducray AD, Schlattner U, Wallimann T, Widmer HR. Functions and effects of creatine in the central nervous system. *Brain Res Bull* 2008; **76**: 329-43.
91. Beal MF. Neuroprotective effects of creatine. *Amino Acids* 2011; **40**: 1305-13.
92. Wilken B, Ramirez J, Probst I, Richter D, Hanefeld F. Anoxic ATP depletion in neonatal mice brainstem is prevented by creatine supplementation. *Arch Dis Child Neonatal Ed* 2000; **82**: F224-F27.
93. Pulido S, Passaquin A, Leijendekker W, Challet C, Wallimann T, Rüegg U. Creatine supplementation improves intracellular Ca 2+ handling and survival in mdx skeletal muscle cells. *FEBS letters* 1998; **439**: 357-62.
94. Malcon C, Kaddurah-Daouk R, Beal MF. Neuroprotective effects of creatine administration against NMDA and malonate toxicity. *Brain Res* 2000; **860**: 195-98.
95. Xu CJ, Klunk WE, Kanfer JN, Xiong Q, Miller G, Pettegrew JW. Phosphocreatine-dependent Glutamate Uptake by Synaptic Vesicles A COMPARISON WITH ATP-DEPENDENT GLUTAMATE UPTAKE. *J Biol Chem* 1996; **271**: 13435-40.

96. Brewer GJ, Wallimann TW. Protective Effect of the Energy Precursor Creatine Against Toxicity of Glutamate and β-Amyloid in Rat Hippocampal Neurons. *J Neurochem* 2000; **74**: 1968-78.
97. Braissant O, Henry H, Béard E, Uldry J. Creatine deficiency syndromes and the importance of creatine synthesis in the brain. *Amino acids* 2011; **40**: 1315-24.
98. Hervias I, Beal MF, Manfredi G. Mitochondrial dysfunction and amyotrophic lateral sclerosis. *Muscle Nerve* 2006; **33**: 598-608.
99. Peters OM, Ghasemi M, Brown RH. Emerging mechanisms of molecular pathology in ALS. *J Clin Invest* 2015; **125**: 1767-79.
100. Zhang W, Narayanan M, Friedlander RM. Additive neuroprotective effects of minocycline with creatine in a mouse model of ALS. *Ann Neurol* 2003; **53**: 267-70.
101. Scott S, Kranz JE, Cole J, Lincecum JM, Thompson K, Kelly N et al. Design, power, and interpretation of studies in the standard murine model of ALS. *Amyotroph Lateral Scler* 2008; **9**: 4-15.
102. Bender A, Samtleben W, Elstner M, Klopstock T. Long-term creatine supplementation is safe in aged patients with Parkinson disease. *Nutr Res* 2008; **28**: 172-78.
103. Investigators NN-P. A randomized, double-blind, futility clinical trial of creatine and minocycline in early Parkinson disease. *Neurology* 2006; **66**: 664-71.
104. Wang J, Xiao Y, Chen S, Zhong C, Luo M, Luo H. Creatine for Parkinson's disease. *Cochrane Libr* 2012.
105. Kieburtz K, Tilley BC, Elm JJ, Babcock D, Hauser R, Ross GW et al. Effect of creatine monohydrate on clinical progression in patients with Parkinson disease: a randomized clinical trial. *Jama* 2015; **313**: 584-93.
106. Li Z, Wang P, Yu Z, Cong Y, Sun H, Zhang J et al. The effect of creatine and coenzyme q10 combination therapy on mild cognitive impairment in Parkinson's disease. *Euro Neurol* 2015; **73**: 205-11.

107. Verbessem P, Lemiere J, Eijnde B, Swinnen S, Vanhees L, Van Leemputte M et al. Creatine supplementation in Huntington's disease A placebo-controlled pilot trial. *Neurology* 2003; **61**: 925-30.

108. Hersch S, Gevorkian S, Marder K, Moskowitz C, Feigin A, Cox M et al. Creatine in Huntington disease is safe, tolerable, bioavailable in brain and reduces serum 8OH2′ dG. *Neurology* 2006; **66**: 250-52.

109. Rosas HD, Doros G, Gevorkian S, Malarick K, Reuter M, Coutu J-P et al. PRECREST: a phase II prevention and biomarker trial of creatine in at-risk Huntington disease. *Neurology* 2014; **82**: 850-57.

110. Braak H, Ghebremedhin E, Rüb U, Bratzke H, Del Tredici K. Stages in the development of Parkinson's disease-related pathology. *Cell Tissue Res* 2004; **318**: 121-34.

111. Bender A, Klopstock T. Creatine for neuroprotection in neurodegenerative disease: end of story? *Amino acids* 2016: 1-12.

112. Casson RJ, Chidlow G, Wood JP, Osborne NN. The effect of hyperglycemia on experimental retinal ischemia. *Arch Ophthal* 2004; **122**: 361-66.

113. Casson RJ, Han G, Ebneter A, Chidlow G, Glihotra J, Newland H et al. Glucose-Induced Temporary Visual Recovery in Primary Open-Angle Glaucoma: A Double-Blind, Randomized Study. *Ophthalmology* 2014; **121**: 1203-11.

114. Nadal-Nicolás FM, Salinas-Navarro M, Vidal-Sanz M, Agudo-Barriuso M. Two methods to trace retinal ganglion cells with fluorogold: from the intact optic nerve or by stereotactic injection into the optic tract. *Exp Eye Res* 2015; **131**: 12-19.

115. Nash MS, Osborne NN. Assessment of Thy-1 mRNA levels as an index of retinal ganglion cell damage. *Invest Ophthalmol Vis Sci* 1999; **40**: 1293-98.

116. Schlamp CL, Johnson EC, Li Y, Morrison JC, Nickells RW. Changes in Thy1 gene expression associated with damaged retinal ganglion cells. *Mol Vis* 2001; **7**: 192-201.

117. Perry V, Morris R, Raisman G. Is Thy-1 expressed only by ganglion cells and their axons in the retina and optic nerve? *J Neurocytol* 1984; **13**: 809-24.
118. Sánchez-Migallón M, Nadal-Nicolás F, Jimenez-Lopez M, Sobrado-Calvo P, Vidal-Sanz M, Agudo-Barriuso M. Brain derived neurotrophic factor maintains Brn3a expression in axotomized rat retinal ganglion cells. *Exp Eye Res* 2011; **92**: 260-67.
119. Slater BJ, Mehrabian Z, Guo Y, Hunter A, Bernstein SL. Rodent anterior ischemic optic neuropathy (rAION) induces regional retinal ganglion cell apoptosis with a unique temporal pattern. *Invest Ophthalmol Vis Sci* 2008; **49**: 3671-76.
120. Kwong JM, Quan A, Kyung H, Piri N, Caprioli J. Quantitative analysis of retinal ganglion cell survival with Rbpms immunolabeling in animal models of optic neuropathies. *Invest Ophthalmol Vis Sci* 2011; **52**: 9694.
121. Lee E-S, Lee J-Y, Jeon C-J. Types and density of calretinin-containing retinal ganglion cells in mouse. *Neurosci Res* 2010; **66**: 141-50.
122. Galindo-Romero C, Harun-Or-Rashid M, Jiménez-López M, Vidal-Sanz M, Agudo-Barriuso M, Hallböök F. Neuroprotection by α2-Adrenergic Receptor Stimulation after Excitotoxic Retinal Injury: A Study of the Total Population of Retinal Ganglion Cells and Their Distribution in the Chicken Retina. *PLoS One* 2016; **11**: e0161862.
123. Gómez-Vicente V, Lax P, Fernández-Sánchez L, Rondón N, Esquiva G, Germain F et al. Neuroprotective effect of tauroursodeoxycholic acid on N-methyl-D-aspartate-induced retinal ganglion cell degeneration. *PLoS One* 2015; **10**: e0137826.
124. Kwong JM, Hoang C, Dukes RT, Yee RW, Gray BD, Pak KY et al. Bis (Zinc-Dipicolylamine), Zn-DPA, a New Marker for ApoptosisZn-DPA, a New Marker for Apoptosis. *Invest Ophthalmol Vis Sci* 2014; **55**: 4913-21.
125. Araki CM, Hamassaki-Britto DE. Calretinin co-localizes with the NMDA receptor subunit NR1 in cholinergic amacrine cells of the rat retina. *Brain Res* 2000; **869**: 220-24.

126. Dreyer EB, Pan Z-H, Storm S, Lipton SA. Greater sensitivity of larger retinal ganglion cells to NMDA-mediated cell death. *Neuroreport* 1994; **5**: 629-31.
127. Schlamp CL, Montgomery AD, Mac Nair CE, Schuart C, Willmer DJ, Nickells RW. Evaluation of the percentage of ganglion cells in the ganglion cell layer of the rodent retina. 2013.
128. Siliprandi R, Canella R, Carmignoto G, Schiavo N, Zanellato A, Zanoni R et al. N-methyl-D-aspartate-induced neurotoxicity in the adult rat retina. *Vis Neurosci* 1992; **8**: 567-73.
129. Vorwerk CK, Kreutz MR, Dreyer EB, Sabel BA. Systemic L-kynurenine administration partially protects against NMDA, but not kainate-induced degeneration of retinal ganglion cells, and reduces visual discrimination deficits in adults rats. *Invest Ophthalmol Vis Sci* 1996; **37**: 2382-92.
130. Huang W, Fileta J, Guo Y, Grosskreutz CL. Downregulation of Thy1 in retinal ganglion cells in experimental glaucoma. *Curr Eye Res* 2006; **31**: 265-71.
131. Baimbridge K, Celio M, Rogers JH. Calcium-binding proteins in the nervous system. *Trends Neurosci* 1992; **15**: 303-08.
132. Ikura M. Calcium binding and conformational response in EF-hand proteins. *Trends Biochem Sci* 1996; **21**: 14-17.
133. Chidlow G, Casson R, Sobrado-Calvo P, Vidal-Sanz M, Osborne NN. Measurement of retinal injury in the rat after optic nerve transection: an RT-PCR study. *Mol Vis* 2005; **11**: 387-96.
134. Berkelaar M, Clarke D, Wang Y, Bray G, Aguayo A. Axotomy results in delayed death and apoptosis of retinal ganglion cells in adult rats. *J Neurosci* 1994; **14**: 4368-74.
135. Galindo-Romero C, Aviles-Trigueros M, Jimenez-Lopez M, Valiente-Soriano F, Salinas-Navarro M, Nadal-Nicolas F et al. Axotomy-induced retinal ganglion cell death in adult mice: quantitative and topographic time course analyses. *Exp Eye Res* 2011; **92**: 377-87.

136. Wang W, Chan A, Qin Y, Kwong JM, Caprioli J, Levinson R et al. Programmed cell death-1 is expressed in large retinal ganglion cells and is upregulated after optic nerve crush. *Exp Eye Res* 2015; **140**: 1-9.

137. Bai N, Aida T, Yanagisawa M, Katou S, Sakimura K, Mishina M et al. NMDA receptor subunits have different roles in NMDA-induced neurotoxicity in the retina. *Mol Brain* 2013; **6**: 34.

138. Selles-Navarro I, Villegas-Perez MP, Salvador-Silva M, Ruiz-Gomez JM, Vidal-Sanz M. Retinal ganglion cell death after different transient periods of pressure-induced ischemia and survival intervals. A quantitative in vivo study. *Invest Ophthalmol Vis Sci* 1996; **37**: 2002-14.

139. Pérez De Sevilla Müller L, Shelley J, Weiler R. Displaced amacrine cells of the mouse retina. *J Comp Neurol* 2007; **505**: 177-89.

140. Dräger U, Olsen JF. Ganglion cell distribution in the retina of the mouse. *Invest Ophthalmol Vis Sci* 1981; **20**: 285-93.

141. Zhang H, Kouadio A, Cartledge D, Godwin AK. Role of gamma-synuclein in microtubule regulation. *Exp Cell Res* 2011; **317**: 1330-39.

142. Nadal-Nicolás FM, Sobrado-Calvo P, Jiménez-López M, Vidal-Sanz M, Agudo-Barriuso M. Long-Term Effect of Optic Nerve Axotomy on the Retinal Ganglion Cell LayerOptic Nerve Axotomy and Ganglion Cell Layer. *Invest Ophthalmol Vis Sci* 2015; **56**: 6095-112.

143. Lam TT, Abler AS, Kwong JM, Tso MO. N-methyl-D-aspartate (NMDA)–induced apoptosis in rat retina. *Invest Ophthalmol Vis Sci* 1999; **40**: 2391-97.

144. Behrend L, Henderson G, Zwacka R. Reactive oxygen species in oncogenic transformation. *Biochem Soc Trans* 2003; **31**: 1441-44.

145. Bruening W, Giasson BI, Klein-Szanto AJ, Lee VMY, Trojanowski JQ, Godwin AK. Synucleins are expressed in the majority of breast and ovarian carcinomas and in preneoplastic lesions of the ovary. *Cancer* 2000; **88**: 2154-63.

146. Ullian E, Barkis W, Chen S, Diamond J, Barres B. Invulnerability of retinal ganglion cells to NMDA excitotoxicity. *Molecular and Cellular Neuroscience* 2004; **26**: 544-57.

147. Ji D, Li G-Y, Osborne NN. Nicotinamide attenuates retinal ischemia and light insults to neurones. *Neurochem Int* 2008; **52**: 786-98.

148. Beal MF, Matthews RT, Tieleman A, Shults CW. Coenzyme Q 10 attenuates the 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) induced loss of striatal dopamine and dopaminergic axons in aged mice. *Brain Res* 1998; **783**: 109-14.

149. Schulz JB, Henshaw DR, Matthews RT, Beal MF. Coenzyme Q 10 and nicotinamide and a free radical spin trap protect against MPTP neurotoxicity. *Exp Neurol* 1995; **132**: 279-83.

150. Nucci C, Tartaglione R, Cerulli A, Mancino R, Spano A, Cavaliere F et al. Retinal damage caused by high intraocular pressure–induced transient ischemia is prevented by coenzyme Q10 in rat. *Int Rev Neurobiol* 2007; **82**: 397-406.

151. Wood JP, Mammone T, Chidlow G, Greenwell T, Casson RJ. Mitochondrial inhibition in rat retinal cell cultures as a model of metabolic compromise: mechanisms of injury and neuroprotection. *Invest Ophthalmol Vis Sci* 2012; **53**: 4897-909.

152. Schuettauf F, Stein T, Choragiewicz TJ, Rejdak R, Bolz S, Zurakowski D et al. Caspase inhibitors protect against NMDA‐mediated retinal ganglion cell death. *Clin Exp Ophthalmol* 2011; **39**: 545-54.

153. Shen H, Goldberg MP. Creatine pretreatment protects cortical axons from energy depletion in vitro. *Neurobiol Dis* 2012; **47**: 184-93.

154. Klivenyi P, Kiaei M, Gardian G, Calingasan NY, Beal MF. Additive neuroprotective effects of creatine and cyclooxygenase 2 inhibitors in a transgenic mouse model of amyotrophic lateral sclerosis. *J Neurochem* 2004; **88**: 576-82.

155. Mittag TW, Danias J, Pohorenec G, Yuan HM, Burakgazi E, Chalmers–Redman R et al. Retinal damage after 3 to 4 months of elevated intraocular pressure in a rat glaucoma model. *Invest Ophthalmol Vis Sci* 2000; **41**: 3451-59.

156. Doh SH, Kim JH, Lee KM, Park HY, Park CK. Retinal ganglion cell death induced by endoplasmic reticulum stress in a chronic glaucoma model. *Brain Res* 2010; **1308**: 158-66.

157. Gu Z, Yamamoto T, Kawase C, Matsubara M, Kawase K, Sawada A et al. Neuroprotective effect of N-methyl-D-aspartate receptor antagonists in an experimental glaucoma model in the rat. *Nippon Ganka Gakkai Zasshi* 2000; **104**: 11-16.

158. Osborne N, Ugarte M, Chao M, Chidlow G, Bae J, Wood J et al. Neuroprotection in relation to retinal ischemia and relevance to glaucoma. *Surv Ophthalmol* 1999; **43**: S102-S28.

159. Sucher NJ, Lipton SA, Dreyer EB. Molecular basis of glutamate toxicity in retinal ganglion cells. *Vision Res* 1997; **37**: 3483-93.

160. Sabel BA, Sautter J, Stoehr T, Siliprandi R. A behavioral model of excitotoxicity: retinal degeneration, loss of vision, and subsequent recovery after intraocular NMDA administration in adult rats. *Exp Brain Res* 1995; **106**: 93-105.

161. Ishikawa M. Abnormalities in glutamate metabolism and excitotoxicity in the retinal diseases. *Scientifica* 2013; **2013**.

162. Chiu K, Lam TT, Li WWY, Caprioli J, Kwong JMK. Calpain and N-methyl-d-aspartate (NMDA)-induced excitotoxicity in rat retinas. *Brain Res* 2005; **1046**: 207-15.

163. Wang Q, Yu S, Simonyi A, Sun GY, Sun AY. Kainic acid-mediated excitotoxicity as a model for neurodegeneration. *Mol Neurobiol* 2005; **31**: 3-16.

164. Yu S, Yeh C-H, Strasser U, Tian M, Choi D. NMDA receptor-mediated K+ efflux and neuronal apoptosis. *Science* 1999; **284**: 336-39.

165. Pivovarova NB, Andrews SB. Calcium-dependent mitochondrial function and dysfunction in neurons. *Febs Journal* 2010; **277**: 3622-36.
166. Chidlow G, Osborne NN. Rat retinal ganglion cell loss caused by kainate, NMDA and ischemia correlates with a reduction in mRNA and protein of Thy-1 and neurofilament light. *Brain Res* 2003; **963**: 298-306.
167. Büchi E, Suivaizdis I, Fu J. Pressure-induced retinal ischemia in rats: an experimental model for quantitative study. *Ophthalmologica* 1991; **203**: 138-47.
168. AliMohammadi M, Eshraghian M, Zarindast M-R, Aliaghaei A, Pishva H. Effects of creatine supplementation on learning, memory retrieval, and apoptosis in an experimental animal model of Alzheimer disease. *Med J Islam Repub Iran* 2015; **29**: 273.
169. Hausmann O, Fouad K, Wallimann T, Schwab M. Protective effects of oral creatine supplementation on spinal cord injury in rats. *Spinal Cord* 2002; **40**: 449-56.
170. Holtzman D, Togliatti A, Khait I, Jensen F. Creatine increases survival and suppresses seizures in the hypoxic immature rat. *Pediatr Res* 1998; **44**: 410-14.
171. Prass K, Royl G, Lindauer U, Freyer D, Megow D, Dirnagl U et al. Improved reperfusion and neuroprotection by creatine in a mouse model of stroke. *J Cereb Blood Flow Metab* 2007; **27**: 452-59.
172. Vis JC, de Boer-van Huizen RT, Verbeek MM, de Waal RM, Hans J, Kremer B. Creatine protects against 3-nitropropionic acid-induced cell death in murine corticostriatal slice cultures. *Brain Res* 2004; **1024**: 16-24.
173. Bull ND, Martin KR. Concise Review: Toward Stem Cell-Based Therapies for Retinal Neurodegenerative Diseases. *Stem Cells* 2011; **29**: 1170-75.
174. Skovronsky DM, Lee VM-Y, Trojanowski JQ. Neurodegenerative diseases: new concepts of pathogenesis and their therapeutic implications. *Annu Rev Pathol Mech Dis* 2006; **1**: 151-70.

175. Deldicque L, Décombaz J, Foncea HZ, Vuichoud J, Poortmans JR, Francaux M. Kinetics of creatine ingested as a food ingredient. *Euro J Appl Physiol* 2008; **102**: 133-43.

176. Solberg Y, Rosner M, Turetz J, Belkin M. MK-801 has neuroprotective and antiproliferative effects in retinal laser injury. *Invest Ophthalmol Vis Sci* 1997; **38**: 1380-89.

177. May CA, Mittag T. Optic nerve degeneration in the DBA/2NNia mouse: is the lamina cribrosa important in the development of glaucomatous optic neuropathy? *Acta Neuropathol* 2006; **111**: 158-67.

178. Vickers J, Schumer R, Podos S, Wang R, Riederer B, Morrison J. Differential vulnerability of neurochemically identified subpopulations of retinal neurons in a monkey model of glaucoma. *Brain Res* 1995; **680**: 23-35.

179. Quigley HA, Nickells RW, Kerrigan LA, Pease ME, Thibault DJ, Zack DJ. Retinal ganglion cell death in experimental glaucoma and after axotomy occurs by apoptosis. *Invest Ophthalmol Vis Sci* 1995; **36**: 774-86.

180. Qu J, Kaufman Y, Washington I. Coenzyme Q10 in the human retina. *Invest Ophthalmol Vis Sci* 2009; **50**: 1814-18.

181. Russo R, Cavaliere F, Rombolà L, Gliozzi M, Cerulli A, Nucci C et al. Rational basis for the development of coenzyme Q10 as a neurotherapeutic agent for retinal protection. *Prog Brain Res* 2008; **173**: 575-82.